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MUDr. Petra Fundová

Mucosal immunity in upper respiratory tract diseases and autoimmunity diseases

Ph.D.Thesis

Supervisor: Prof. MUDr. Helena Tlaskalová-Hogenová, DrSc.

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ABSTRACT

Mucosal immune system comprises not only the major compartment of the immune system but also important interface with the outer environment. It is responsible in maintaining an intricate balance with the danger and non-danger stimuli of the outer world by employing specific anatomical features and unique functional mechanisms. Mucosal immune system has been long understudied, perhaps due to the limited accessibility, and its biological importance is thus still underevaluated. However, it has become evident that it is important to study mucosal immune system not only in local mucosal affections but also when uncovering pathogenic mechanisms and novel prevention strategies of organ specific autoimmune diseases such as type 1 diabetes.

Thus, the first, more clinically oriented part of this thesis is focused on mucosal immune system of the upper respiratory tract in disease conditions - in nasal polyposis (NP).

Because there is a substantial accumulation of eosinophils and neutrophils in the most frequent type of NP, we investigated and described increased expression of chemokine receptors CCR1 and CCR3 in NP versus nasal mucosa. Both innate immune mechanisms as well as homeostasis of epithelial cells may participate in NP. We have documented increased numbers of iNOS-positive and insulin-like growth factor-1 receptor (IGF-1R)-positive cells in both stroma and epithelium of NP.

The second part of the thesis deals with the interplay of the mucosal immune system, dietary gluten or gliadin in pathogenesis and a novel prevention strategy of autoimmune type 1 diabetes (T1D).

Because gluten-free (GF) diets highly prevent T1D in animal models we investigated the effect of dietary gluten on the mouse mucosal immune system. In the first two papers, we compared the effects of the standard, diabetes permissive vs. the diabetes preventive GF diet and found changes in proportion of mucosal $\gamma\delta$ T cells, Th17 cells as well as a more proinflammatory cytokine signature associated with the standard, diabetes-permissive diet. Since not many studies addressed in detail the innate immune mechanisms of gliadin we next investigated innate signaling pathways utilized by gliadin fragments. We documented that apart from IL-1β induction, TLR2/4/MyD88/TRIF/MAPK/NF-κB innate signaling pathway and NLRP3 inflammasome activation are involved in wheat proteins signaling. Finally, we tested intranasal (i.n.) administration of gliadin as a novel vaccination strategy - using an environmental compound that may have etiological role in T1D. We showed that i.n. gliadin is able to substantially reduce diabetes incidence in NOD mice and also reduce diabetes incidence when applied much later to already prediabetic mice. The effect was associated with increased proportion of potentially regulatory $\gamma\delta$ T cells, and to a lesser extent also CD4$^+$Foxp$^+$ Tregs, in mucosal compartments as well as with changes in their cytokine signatures.

In conclusion, this thesis deals with the mucosal immune system. We addressed some pathogenic processes in a local mucosal affection - NP as well as studied interactions of environmental factors and mucosal immune system in pathogenesis and also as possible novel prevention strategy in T1D.

Keywords: mucosal immune system; nasal polyposis; type 1 diabetes; pathogenesis; prevention.

ABSTRAKT

Slizniční imunitní systém představuje nejen hlavní složku imunitního systému, ale také důležité rozhraní s vnějším prostředím. Je zodpovědný za udržování jemné rovnováhy mezi bezpečnými a nebezpečnými podněty z okolí za využití specifických anatomických struktur a funkčních mechanismů. Slizniční imunitní systém nebyl dlouho dostatečně studován, také kvůli své omezené dostupnosti, a jeho biologický význam je podceňován. Nicméně je zřejmé, že jeho studium je důležité nejen v místních interakcích na sliznicích, ale také při odhalování patogenetických mechanizmů a nových strategií prevence orgánově specifických autoimunitních onemocnění jako například T1D.

První, více klinicky orientovaná část této disertace je zaměřena na slizniční imunitní systém horního respiračního traktu postižený chorobou – nosní polypózou (NP). Vzhledem k tomu, že nejčastěji se vyskytují polypy se značnou akumulací eosinofilů a neutrofilů, vyšetřovali jsme a popsali zvýšenou expresi chemokinových receptorů CCR1 a CCR3 v nosních polypech oproti nosní sliznici. K nosní polypoze mohou přispívat jak mechanismy přirozené imunity, tak homeostáza epitelových buněk. Zdokumentovali jsme zvýšený počet iNOS pozitivních a insulin-like growth factor-1 receptor (IGF-1R) pozitivních buněk jak ve stromatu, tak v epitelu NP.

Druhá část práce se zabývá souhrou slizničního imunitního systému, glutenu či gliadinu z diety v patogenezi a v nové strategii prvence autoimunitního diabetu 1. typu (T1D). Protože bezlepková (GF) dieta u zvířecích modelů vysoce zabraňuje T1D, studovali jsme efekt glutenu z diety na myší slizniční imunitní systém. V prvních dvou publikacích jsme srovnávali efekt standartní, diabetes-permisivní s diabetes preventivní GF dietou a našli jsme rozdíly v proporci slizničních γδ T buněk, Th17 buněk stejně jako zvýšenou produkci prozánětlivých cytokinů spojených se standartní, diabetes permisivní dietou. Protože pouze málo studií se zabývalo detailně mechanismy přirozené imunity u gliadinu, jako další jsme vyšetřovali signalizační dráhy přirozené imunity užívané fragmenty gliadinu. Zdokumentovali jsme, že kromě indukce IL-1β, jsou v signalizaci pšeničných proteinů zahrnuty i TLR2/4/MyD88/TRIF/MAPK/NF-κB signalizační dráha a aktivace inflamazomu NLRP3. Nakonec jsme testovali intranasální (i.n.) administraci gliadinu jako novou vakcinační strategii – užití složky prostředí, která může hrát roli v etiologii T1D. Ukázali jsme, že i.n.gliadin je schopen značně redukovat incidenci diabetu u NOD myší a také redukuje incidenci diabetu, když je podán mnohem později již prediabetickým myším. Tento efekt byl spojován se zvýšeným podílem potenciálně regulačních γδ T buněk, a v menší míře také CD4+Foxp+Tregs ve slizničních kompartmentech, stejně jako se změnou cytokinových profilů.

Závěrem, tato práce se zabývá slizničním imunitním systémem. Zaměřili jsme se na určité patogenetické procesy v místních slizničních vztazích - NP a současně jsme studovali interakce mezi faktory vnějšího prostředí a slizničního imunitního systému v patogenezi a také v možné novátorské prevenční strategii u T1D.

Klíčová slova: slizniční imunitní systém; nosní polypóza; diabetes 1. typu; patogeneze; prevence.

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ABBREVIATIONS

AI	Amylase inhibitor
ANOVA	Analysis of variance
APC	Antigen-presenting cell
Apo-E	Apolipoprotein E
ARS	Acute rhinosinusitis
ASC	Apoptosis-associated speck like protein
ASL	Airway surface liquid
ATP	Adenosine triphosphate
BB	Biobreeding
BMDC	Bone marrow derived dendritic cells
CCL	C-C chemokine ligands
CCR1	C-C chemokine receptor type 1
CCR3	C-C chemokine receptor type 3
CD	Celiac disease
CD	Cluster of differentiation
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CNS	Central nervous system
CRS	Chronic rhinosinusitis
CRSsNP	CRS without nasal polyps
CRSwNP	CRS with nasal polyps
CXCR3	CXC chemokine receptor 3
CY3	Indocarbocyanine
DCs	Dendritic cells
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonukleová kyselina
DTT	Dithiothreitol
EAE	Autoimmune encephalomyelitis
EBV	Epstein-Barr virus

EGF	Epidermal growth factor
ELISA	Enzyme-Linked ImmunoSorbent Assay
ENT	Ear-nose-throat
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
Fc	Antibody fragment crystallizable
FESS	Functional endoscopic sinus surgery
FITC	Fluorescein isocyanate
Foxp3	Forkhead box P3
FSC	Forward-scattered light
GAD	glutamic acid decarboxylase
GF	Gluten-free
GM-CSF	Granulocyte/macrophage colony-stimulating factor
H&E	Hematoxylin & eosin
HLA	Human leukocyte antigen
ICAM-1,-2	Intercellular adhesion molecule -1, -2
IBD	Idiopathic inflammatory bowel diseases
IEL	Intraepithelial lymphocytes
IFN-γ	Interferon gamma
IGF-1R	Insulin-like growth factor-1 receptor
IgG	Immunoglobulin G
IgG F	Immunoglobulin G Fragment
IL	Interleukin
ILN	Inguinal lymph nodes
i.n.	Intranasal
JNK	Jun amino-terminal kinases
KCl	Chlorid draselný
kDa	Kilo Dalton
KGF	Keratinocyte growth factor
LPS	Lipopolysaccharide
iNOS	Inducible nitric oxide synthase

mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCP-3	Monocyte chemoattractant protein 3
MCP-4	Monocyte chemoattractant protein 4
MEC	Mucosae-associated epithelial chemokine
MHC	Hlavní histokompatibilní komplex
MIP-1 α	Macrophage inflammatory protein 1α
MIP-3	Macrophage inflammatory protein-3
MLN	Mesenteric lymph nodes
mM	mili Molar
MyD88	Myeloid differentiation primary response gene 88
NAC	N-Acetyl-L-cysteine
NALT	Nasal-associated lymphoid tissue
NFκ-B	Nuclear factor κB
NK cell	Natural killer cell
NLRP1,3	Nod-like receptor family containing pyrin domain 1,3
NO	Nitric oxide
NOD	Nucleotide Oligomerization Domain
NOD mice	Non-obese diabetic mice
NP	Nasal polyps
NM	Nasal mucosa
OCT	Optimal cutting temperature
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PDWGF	Pepsin digest of wheat gliadin fraction
PLN	Pancreatic lymph node
PMA	Phorbol myristate acetate
PmB	Phenylmercuric borate
PP	Peyers Patches
PRR	pattern recognition receptor
P2X7	purinoceptor 7

RANTES	Regulated upon activation in normal T cells expressed and secreted protein
RIG	Retinoic acid-inducible gene
RNA	Ribonucleic acid
RORγ	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RS	Rhinosinusitis
S	Spleen
SEM	Standard error of mean
SIgA	Secretory immunoglobulin A
SNP	Single nucleotide polymorphism
SPF	Specific Pathogen Free
SSC	Side-scattered light
STD	Standard
TBS	TRIS buffered saline
Teff	Effector T cells
TGF-β	Transforming growth factor beta
Th cell	T helper cell
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
TPCK	N-p-Tosyl-L-phenyl-alanine chloromethyl ketone
Tregs	Regulatory T cells
TRIF	Toll-interleukin-1 receptor domain-containing adaptor-inducing interferon-β
T1D	Type 1 diabetes mellitus
VCAM-1	Vascular cell adhesion molecule 1
μM	Micro Molar

1. INTRODUCTION

1.1. Mucosal immune system

1.1.1. Characteristics

The mucosal immune system comprises from upper and lower respiratory tract, gastrointestinal tract, urogenital tract, olfactory mucosa and duct portions of the exocrine glands including mammary glands. The total mucosal surface area in the body reaches more than 300 m^2, while area of the skin reaches in adult about 1.5 m^2. Mucosal lymphatic tissue is in fact the biggest immune organ in the body containing in a healthy adult around 80% of immunocompetent cells of the body. The mucosal immune system displays several unique anatomical structures as well functional mechanisms, that have most likely devolved due to the specific interaction with the outer environment, including commensal microflora, present on many mucosal surfaces (Brandtzaeg et al., 2008).

The mucosal immune system has following main functions: (1) anti-infectious role - protection of the mucous membranes against colonization and invasion by potentially dangerous microbes that may be encountered, (2) barrier function - barrier against penetration of infectious and immunogenic components present on the mucosas into the circulation and thus into the inner environment of the organism (3) 'oral or mucosal tolerance' - low reactivity or immunosupression to harmless antigens present on mucosal surfaces (4) immunoregulatory function - maintenance of mucosal homeostasis and to prevent uptake of non-degraded antigens including foreign proteins derived from ingested food, airborne matter and commensal microorganisms, as well as to prevent the development of potentially harmful immune responses to these antigens if they do reach the body interior (Tlaskalová-Hogenová et al., 2002; Holmgren and Czerkinsky, 2005; McGhee and Fujihashi, 2012).

Thus, in relation to the above listed functions, several mechanisms within the mucosal immune system are enhanced or uniquely developed. Mucosal surfaces are characterized by well developed barrier function – that is achieved by the presence of a mucus layer consisting of complex of mucins and glycoproteins, local production of antibacterial agents by mucosa (e.g. defensins, collectins), the continuous layer of rapidly renewing epithelial cells and their interconnection by tight junctions (Ogra et al., 1999; Tlaskalová-Hogenová et al., 1995). Last but not least, mucosal immune system is also

characterized by the specific feature - production of secretory immunoglobulins, IgA but also IgM, that are produced by plasma cells as polymers and transported via specific polymeric immunoglobulin receptor from basolateral site of the epithelial cells (e.g. enterocytes) to luminal surfaces. This barrier function of mucosal surfaces is neonatally subsidized by secretory IgA present in milk and colostrum (Ogra et al. 1999; Brandzaeg, 2009).

Another feature of the mucosal immune system is represented by strong innate immune (Kumar et al., 2011; Santaoalla et al., 2011) mechanisms that has to deal with the imminent danger signals as well as the interplay with the nervous systems and impacts of neuro-messengers (Alonso et al., 2014; Assas et al., 2014).

1.1.2. Inductive and effector sites of the mucosal immune system

The mucosal immune system can principally be divided into inductive sites - where antigens sampled from mucosal surfaces stimulate cognate naive T and B lymphocytes - and effector sites - where the effector cells after extravasation, retention, and differentiation perform their action, for instance by contributing to the formation of secretory antibodies (Brandtzaeg et al., 2008, Brandtzaeg et al., 2009; McGhee and Fujihashi, 2012).

The inductive sites for mucosal immunity are represented by organized mucosa-associated lymphoid tissue (MALT) as well as local/regional mucosa-draining lymph nodes (LNs). All MALT structures resemble LNs with variable T-cell zones intervening between the B-cell follicles and contain a variety of antigen-presenting cells, including DCs and macrophages. However, MALT lacks afferent lymphatics because all such lymphoid structures actively sample exogenous antigens directly from the mucosal surfaces through a characteristic follicle-associated epithelium containing "microfold" or "membrane" (M) cells. (Ogra et al., 1999; Brandtzaeg et al., 2008). Thus, the M cells are highly specialized epithelial cells that transport transepithelialy soluble and especially particulate antigens such as macromolecules, particles and microorganisms from the gut lumen. (Neutra et al., 1996)

The MALT represents a highly compartmentalized immunological system and functions essentially independent from the systemic immune apparatus (Holmgren and Czerkinsky, 2005). It is comprised of anatomically defined lymphoid microcompartments such as NALT - nasopharynx associated lymphoid tissue, cervical lymph nodes, GALT - gut associated lymphoid tissue comprising the PP-Peyer patches, MLN- the mesenteric

lymph nodes, the appendix and ILF- isolated (solitary) lymphoid follicles in the intestine as well as other mucosal solitary lymph nodes. Some of the structures were sometime specifically named e.g. LALT - larynx-associated lymphoid tissue or TALT (Eustachian tube-associated lymphoid tissue), (Brandtzaeg et al., 2008). Apart from GALT also bronchus-associated lymphoid tissue (BALT) has been reported, however Tschernig and Pabst have shown that BALT is normally found only in infants and under pathological conditions but not in healthy adult humans (Tschernig and Pabst, 2000).
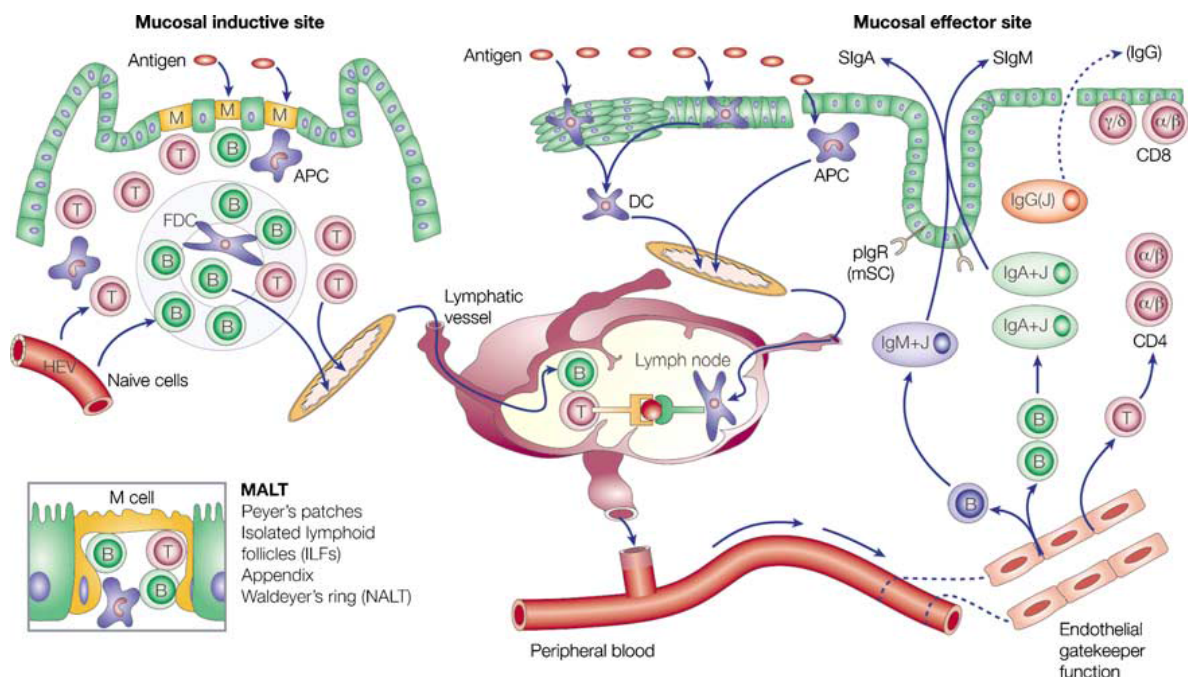

Figure 1. Mucosal inductive and effector sites in mucosal immunity (adapted from Brandtzaeg and Pabst, 2004).

The effector sites consist of distinctly different histological compartments such as the lamina propria (LP) of various mucosae, the stroma of exocrine glands, and surface epithelia (Figure 1; Brandtzaeg and Pabst, 2004; Brandtzaeg et al., 2008). The cellular components of the effect sites of the mucosal immune system and their soluble factors could belong to both the innate as well as adaptive branches of immunity and their involvement may differ according to the anatomical location. Thus, epithelial cells, macrophages, dendritic cells, granulocytes, mast cells but also fibroblasts, NK cells, innate lymphoid cells (ILCs) could be listed as innate components within the effector sites while intraepithelial lymphocytes (including the specific CD8$\alpha\alpha$ homodimer T cells) and lamina

propria lymphocytes belong to the adaptive arm of the mucosal effector sites (Tlaskalová-Hogenová et al., 2002).

1.1.2.1. NALT - nasopharynx (or nasal) -associated lymphoid tissue

The NALT – nasopharynx-associated lymphoid tissue in rodents, which is an equivalent of lymphoid tissue of Waldeyer's pharyngeal ring including the adenoids and palatal tonsils in humans in the upper respiratory tract, is less studied nevertheless important compartment of mucosal immune system, with several connections to both intranasal administration of antigens as well as nasal mucosa itself. There is a bit of confusion in the nomenclature (Brandtzaeg et al., 2008) as NALT has also been often refereed as Nasal- or Nose-associated lymphoid tissue. We and others have shown that NALT is a mucosal inductive site for both humoral and cellular immune responses (Zuercher et al., 2002; Wu et al., 1996). NALT displays several similarities with Peyer's patches (PP) of the gut (Heritage et al. 1997). Naive CD4 cells within NALT display a Th0 phenotype (Hiroi et al., 1998) and have a potential to develop towards regulatory Th2 cells. NALT consist of Bcell zones and T cell areeas consisiting of major populations of navive B cells and naïve T cells (CD45RB^{high}), respectivelly. Its epithelium contains specilized M cells and folloce associated epithelim for antigen uptake (Wu et al., 1996; Takata et al. 2000; Jeong et al., 1999). Intranasal application route has been shown to induce strong mucosal specific but also systenic immune antibody and CTL responses (Heritage et al. 1997; Porgador et al., 1997). NALT high endothelail venus express a specific peripheral node addressin (PNAd) in conjuction with the MAdCAM-1, what is one of the major differences of NALT from PP in the GALT. Thus, slightly different recruitment and retention of the L-seletin expressing naïve lymphocytes occurs in the NALT (Csecsits et al.; 1999).

In conclusion, NALT is an important lymphoid organ to study in induction of immune responses by nasally administered antigens (Daniel and Wegmann, 1996; Harrison et al., 1996; Zanvit et al., 2010).

1.1.3. Microbiota of mucosal surfaces

The skin and mucosal surfaces of the body of vertebrates are colonized by vast numbers of microorganisms, commonly referred as microbiota (Kamada et al., 2013). The microbiota comprises mainly bacteria; however, viruses, fungi and protozoas are also present. Human microbiota consist of trillions of bacterial cells, 10-times more cells than the number of

cells constituting the human body. Some metagenomic studies have suggested that less than 10% of the cells that comprise our bodies are *Homo sapiens* cells. The remaining 90% are bacterial cells. (Hutter et al., 2015). Bacteria are present at anatomical locations that provide suitable conditions for their growth and proliferation. Skin is predominantly colonized by bacteria in the skin folds. The upper airways, particularly the nasopharynx, harbor bacteria, as do some mucosal surfaces of the genital tract, although the greatest number of bacterial cells is found in the digestive tract. The oral cavity (tongue, teeth and periodontal tissues) harbors high numbers of bacteria (10^{12}). The stomach has only 10^3-10^4 bacteria, the jejunum harbors 10^5-10^6 bacteria and the terminal ileum harbors 10^8-10^9. However, the largest number of bacterial cells is found in the large intestine (10^{11} per gram of intestinal content), (Tlaskalová-Hogenová et al., 2011).

Commensal bacteria are indispensable for the normal function of the mammalian organism. It is well known that our ability to extract energy and nutrients from food depends on the presence of resident gut bacteria, the intestinal microbiota (Ivanov and Littman, 2011). Most of the commensal bacteria are symbiotic; however, after translocation through the mucosa or under specific conditions, such as immunodeficiency, commensal bacteria could cause pathology (Tlaskalová-Hogenová et al., 2011). Microbiota represents a complex ecosystem with enormous microbial diversity (Bäckhed et al., 2012). Commensal bacteria are not simply beneficial bystanders, but are important modulators of intestinal immune homeostasis and that the composition of the microbiota is a major factor in pre-determining the type and robustness of mucosal immune responses (Ivanov and Littman, 2011). It is noteworthy that the number of genes of our colonic microbiota exceeds the number of genes in the human genome by 150 times. There are more than 50 bacterial phyla on Earth, but human gut-associated microbiota are dominated by four main phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (Eckburg et al., 2005). Fundamental comparative studies of human fecal microbiota have revealed the astonishing fact that each of us has a unique microbiota, i.e. there are considerable differences between the compositions of the microbiota of individuals (Human Microbiome Project Consortium, 2012). It has also been shown that the main bacterial populations comprising our microbiota stabilize during the first years of life. During this time, the microbiota develops and subsequently remains stable throughout our life in terms of the major bacterial populations, even after antibiotic treatments (Hooper and Macpherson, 2010). Dietary interventions and the administration of probiotics could be effective means of changing the composition of the relatively simple microbial community present in the small intestine and

could thereby substantially affect this community's metabolic and immunomodulatory functions (Tlaskalová-Hogenová et al., 2011).

1.1.4. Common mucosal immune system

The concept of common mucosal immune system has evolved as researchers have noticed that immune responses built upon local mucosal stimulation or induction of immune response could be also detected not only in circulation, but more importantly also in other mucosal sites, including exocrine glands (McDermott and Bienenstock, 1979; Mestecky, 1987; Salmi and Jalkanen, 1999). Thus, upon encounter of an environmental antigen, adaptive immune responses are detected within the effector sites of the whole mucosal immune system.

The molecular basis of the process has become partly uncovered. One of the major contributing mechanism is based on expression of β7 integrins e.g. α4β7 or αEβ7 by mucosal lymphocytes and expression of corresponding homing receptors - mucosal addressin cell adhesion molecule, MAdCAM1 on endothelial cells of mucosal vessels (Shaw and Brenner, 1995).

The concept of common mucosal immune system in distribution of adaptive immune responses within the mucosal compartment was documented for both plasma cells as well as T cells (Ogra et al., 1999). Immune responses within the common mucosal system nevertheless seem to be modified by tissue specificities or by tissue prevailing types of immune responses. Thus, upon antigen encounter, induction of slightly different, tissue-specific immune responses could be found in various mucosal sub-compartments (Zuercher et al., 2002; Matzinger and Kamala, 2011). The common mucosal immune system and the mucosa-wide distribution of adaptive immune responses have not only important physiological role but could be also exploited in vaccination or in other immuno-intervention strategies (Zuercher et al. 2002; Mlckova et al. 2005; Funda et al., 2014; Harrison et al., 1996).

1.1.5. Mucosal tolerance and vaccination

Oral tolerance refers to physiologic induction of tolerance that occurs in the GALT and more broadly at other mucosal surfaces such as the respiratory tract (Faria and Weiner, 2005; Mora et al., 2006; Weiner et al., 2005). Although strong effector immunity is required to protect from pathogens, it is important that other non-dangerous antigens such

dietary components or commensal microflora do not lead to chronic, pathological inflammatory condition. In order to maintain this balance, several mechanisms have evolved both on the effector and regulatory arm of the immune reponse. While "danger" signaling via e.g. pattern recognition receptors is needed for mounting effector immune mechanisms, other antigens (especially native forms) are processed and reacted to in a manner that leads to hyporesponsiveness, also called mucosal (oral) tolerance. The phenomenon of "oral tolerance" has been known for over a century, i.e. hyporesponsiveness to a fed antigen on subsequent challenge with that antigen (Wells and Osborne, 1911; reviewed in Faria and Weiner, 2005). It is now recognized that there are multiple mechanisms of oral tolerance, and one of the prime determinants is the dose of the antigen. Low doses favor the induction of Tregs, whereas higher doses favor the induction of anergy or deletion. High doses of antigen are also needed to suppress humoral immunity. These mechanisms are not exclusive, especially at higher doses. The immunologic mechanisms of oral tolerance have been studied by a many investigators over the past 40 years, and during that time, the immunologic concept of active immune regulation or 'suppression' has been in and out of favor (Faria and Weiner, 2005; Weiner et al., 2011). Both cell to cell interactions as well as soluble factors e.g. anti-inflammatory cytokines (bystander suppression) are involved in the mechanisms of mucosal tolerance. Moreover, it is not only antigen dose or only the cytokine environment within the mucosa, but also antigen presentation and sequence of exposure to cytokines what may determine the outcome of an immune response in mucosal compartment (Mowat et al. 2003; Mowat and Bain, 2011; Li and Flawell, 2008).

The existence of common mucosal immune system together with the ease of administration (oral, nasal) makes mucosal vaccination an ideal application route for induction of protective immune responses. Thus, delivery of vaccines to GALT or NALT may induce systemic responses, providing one can tackle the obstacle of mucosal tolerance (Ennis et al.; 1982; Gallichan and Rosenthal, 1996; Prokešová et al., 2009). For this purpose several mucosal adjuvants have been developed e.g. cholera toxin B (Bergquist et al., 1997).

Indeed, different situation is in prevention of autoimmune diseases, where the phenomenon of mucosal tolerance could be exploited to downregulate autoimmune responses to disease specific antigens or so-called autoantigens (Tarkowski et al., 1999; Harrison et al., 1996; Daniel and Wegmann, 1996; Ramiya et al. 1997). Nevertheless, this

is much more difficult to achieve in humans than in animal models of autoimmune diseases (Hanninen and Harrison, 2004).

1.2. Nasal polyposis

1.2.1. Introduction

Nasal polyposis (NP) is a chronic and recurrent inflammatory disease of nasal and sinus mucosa manifesting by non-tumorous grape-like structures originating from the ostiomeatal complex and prolabing into the upper nasal cavity. It is remarkable that polyps develop exclusively from a few square cm of airway mucous membrane which often is universally inflamed. The reason for this is unknown and one can only speculate about the nature of a "localisation factor". (Mygind et al., 2000). The Hadley's clinical scoring of nasal polyposis (Hadley and Schaefer, 1997) includes categorization of nasal polyps in relation to their sizes. These have been subdivided into 4 stages. It could range from a single solitary polyp to diffuse nasal and paranasal polyposis filling the whole nasal cavity and sinuses, in extreme cases even prolabing from the front of the nostrils or deforming the skeleton of the nose forming a frog-like nose. This leads to nasal obstruction and blockage, nasal discharge, post nasal drip, facial pain, increased sense of smell, dysomia, or anosmia and in general to decreased quality of life (Wabnitz et al., 2005).

1.2.2. Histology

Polyps are characterized by massive tissue edema with sparse fibrous cells, decreased number of mucous glands and vessels with no innervation, proliferation of stromal and epithelial elements and a thickening of the basement membrane (Taylor, 1963). Stroma of nasal polyps consists (except of tissue edema) of supporting fibroblasts and infiltrating cells. Approximately 80% of the infiltrating cells constitute of activated eosinophils (Stoop et al., 1993), followed by neutrophils, mast cells and lymphocytes with increased proportion of T-lymphocytes (Jones et al., 1987; Fokkens et al., 2007). Eosinophils are located along the vessels, mucous glands and under the basement membrane (Kakoi and Hiraide, 1987). Most of the polyp surface is covered by a ciliated pseudostratified epithelium, but a transitional and squamous epithelium is also found, especially in anterior polyps, influenced by the inhaled air currents (Mygind et al., 2000; Larsen and Tos, 1990).

As mentioned above, the cellular components comprise a variety of cells including eosinophils, mast cells, lymphocytes, neutrophils and plasma cells.

Based on histological characteristics, polyps may be broadly classified into four types: (1) eosinophilic edematous type (edematous stroma with a large number of eosinophils); (2) chronic inflammatory or fibrotic type (large number of inflammatory cells mainly lymphocytes and neutrophils with fewer eosinophils); (3) seromucinous gland type; and (4) atypical stromal type. Of the four histological types, the eosinophilic edematous type and the chronic inflammatory type are most commonly seen in clinical practice (Pawankar, 2003). However, this does not hold true in East Asia including China, Korea and Japan, where according many studies eosinophilic NPs are less frequent (Cao et al., 2009; Ikeda et al., 2013, Kim et al., 2011, Kim et al., 2007; Kim et al., 2013; Kato, 2015; Wen et al., 2012; Yoshimura et al., 2011; Zhang et al., 2008).

1.2.3. History

The first reference about nasal polyps is documented in the medical and surgical scripts and scrolls from Ancient Egypt from around 2475 B.C., (Pahor and Farid, 2003). The other references come from the Byzantine times (AD 324-1453). The original Greek-language texts of the Byzantine medical writers describe the early knowledge of the definition, symptoms, conservative treatments, and surgical intervention in cases of this disease. A considerable number of conservative treatments, etiologic and local (with inunctions or blowing of caustic substances), with evident influence from Roman medicine, were identified even in the early Byzantine medical texts (4th century). The first meticulous intranasal surgical removal of polyps was described. During the early 20th century there the effectiveness of various conservative treatments such as radium therapy (Findlay, 1947) or procaine penicillin solutions (Ruskin, 1950) was tested. Around the half of 20th century arose use of the first corticoid aerosols (Lecco, 1953). The surgical treatment for nasal polyposis developed gradually from the 19th century. It wasn't until the late 1960s and 70s, Prof. Walter Messerklinger and Prof. Heinz Stammberger, started what would become a revolution in diagnosis and treatment of nasal and sinuses diseases: rigid endoscopy, functional endoscopic sinus surgery (FESS) being the best known off-spring of this development. The best available treatment for nasal polyposis to date combines both conservative and surgical approach.

1.2.4. Prevalence

Rhinosinusitis (RS) and chronic rhinosinusitis (CRS) are a significant health problem which seems to mirror the increasing frequency of allergic rhinitis and which results in a large financial burden on society (Bachert et al., 2014). Rhinosinusitis is a broad umbrella term covering multiple disease entities, including acute RS (ARS), CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP), (Fokkens et al., 2012). The GA2LEN study concluded that the overall prevalence of CRS by EP3OS criteria was 10.9% (range 6.9- 27.1) (Hastan et al., 2011). Nasal polyposis prevalence ranks from 0.5 till 4.5% and differs in particular geographical regions and most probably is also dependent on genetic predisposition in different races and populations. The prevalence rises with the age and it is more common in men (2.1:1) (Johanson at al., 2003) with exception of Samter's trias (Buchheit and Laidlaw, 2016), in which women are more commonly affected (2.3:1) (Szczeklik, 2000). According the nationwide survey in Korea nasal polyps prevalence diagnosed by nasal endoscopy was 0.5% in the population, while prevalence of CRS was 1.01% (Min, 1996). In following study by Kim in 2011 the prevalence of CRS in Korea was 6.95%. (Kim, 2011). Swedish population study which uses nasal endoscopy for nasal polyps diagnosis states prevalence of the diseases 2.7% in the general population with higher incidence in men and asthmatics (Johanson 2003). Finish study based on questionnaire states prevalence of 4.3 % in adult population (Hedman, 1999). Retrospective study evaluating data of 6000 patients from hospitals, allergy clinics and outpatients offices in the USA states prevalence of 4.2% in general population and 6.7% in asthmatics, respectively (Settipane, 1977). According to the newer studies, rhinosinusitis is highly prevalent in the United States, affecting an estimated 16 % of the adult population annually according to a National Health Interview Survey without specifying the particular disease entities (Anand, 2004). Occurrence of NP in children is rare with the exception in cystic fibrosis patients (Sanders 2016).

1.2.5. Genetic factors and association with other diseases

Family burden is common in nasal polyposis. In a French multicentre study it was stated by 58.7% patients that they have one or more first-degree relatives (parents, full siblings, children) affected by NP; 43.6% had one or more first-degree relatives affected by bronchial asthma (Crampette et al., 2001). Similarly to autoimmune diseases, association with particular HLA alleles was described in NP. For instance, a Hungarian study

comparing 50 patients with NP with 50 healthy individuals showed 2-3 times higher probability of NP development in individuals bearing HLA-DR7-DQA1*0201 and DQB1*0202 (Molnar-Gabor et al. 2000), while a Mexican study on a cohort of 31 patients with NP compared to 151 healthy individuals stated 5.53 times higher risk of disease development in individuals bearing allele HLA-DR7-DQA1*0201 and DQB1*0201 (Fajardo-Dolci et al., 2006). According to the Chinese study comparing HLA alleles in 31 patients with NP to 81 healthy individuals the alleles HLA-DR16, HLA-DQ8 and HLA-DQ9 pose for their bearers an increased risk of disease, while a dominant HLA-DQ7 could be on the contrary connected with higher resistance to the disease (Zhai et al., 2007). Except HLA associations and elevated family burden there was no other strong genetic factor and thus for example a gene therapy will not be a probable future causal therapy of NP. In general, it can be concluded that environmental factors play a crucial role in the development of NP in genetically predisposed individuals.

The population studies show that NP occurs more frequently in patients with bronchial asthma (Hedman et al., 1999, Settipane et al., 1977, Beule 2015). In studies covering large cohorts of patients with NP, asthma was found in 30-60% (Slavin, 1982; Larsen, 1996). Nonspecific bronchial reactivity (BHR) and/or asthma occur more often in patients with NP than in the general population (Slavin, 1982). Allergy as a possible cause of nasal polyposis was proposed by Younge in his original paper published in BMJ in 1907 (Younge, 1907). This paper was cited many times since the time of the publication, however the later studies have not confirmed this very widespread theory (Mygind, 2000). Aspirin intolerance associated with bronchial asthma and nasal polyps is a distinct clinical entity called aspirin, or Samter's or Widal trias (Samter et al., 1967, 1968). Usually, such a triad starts off with vasomotor rhinitis associated with profuse rhinorrhea, followed by severe nasal congestion, development of nasal polyposis, bronchial asthma, and finally aspirin sensitivity. (Pawankar, 2003). It occurs with a prevalence of 10-20% in asthmatic population (Hedman et al, 1999; Samter et al., 1968; Kasper et al., 2003, Simon et al., 2015). In women, who prevailed over the men in ratio 2.3:1, the onset of symptoms is manifested significantly earlier and the disease is also more progressive and severe than in men (Szczeklik et al., 2000, Simon et al., 2015). Differences in HLA genes are considered to be one of the reasons for the high prevalence of polyposis in ASA (Molnar-Gabor et al., 2000). The precise etiology of aspirin-sensitive NP is unclear, but there is an evidence that impairment of prostanoid metabolism could be involved in the pathogenesis of nasal polyps (Mullol et al., 2002). Confirmed associations have also been described between

eosinophilic nasal polyposis and Churg-Strauss syndrome, a form of eosinophilic immunovasculitis (Settipane, 1996; Bachert et al., 2003).

Cystic fibrosis is the most common lethal autosomal recessive disease in Caucasian population (Henriksson, 2002). There has been immense progress in the elucidation of the molecular and cellular pathophysiology of cystic fibrosis (CF) since the cloning of the CF gene in 1989 (Kerem et al., 1989; Riordan et al., 1989). CF reflects the absence of functional CFTR protein at the proper cellular location (Welsh and Smith, 1993). Classifications of CFTR mutations have been developed, that encompass the spectrum of genetic mutations, but the majority appear to involve misfolding of CFTR protein. (Cheng et al., 1990). Recent data have linked the abnormal ion transport properties of CF airway epithelia to depleted airway surface liquid (ASL) volume, reflecting the combined defects of accelerated Na^+ transport and the failure to secrete Cl^- (Boucher, 2004). In the ear-nose-throat area it manifests by chronic sinusitis and nasal polyps. Prevalence of NP in patients with cystic fibrosis is estimated from 7 to 56% with higher prevalence when using the nasal endoscopy (Cepero, 1987; Taylor et al., 1974, Brihaye et al., 1994; Jones et al., 1993, Liang et al., 2013). Dornase alfa and, to a lesser extent, topical steroids demonstrated significant benefits in the medical treatment CRS in CF. There was a lack of evidence to support antibiotic therapy in the outcomes assessed (Liang et al., 2014). In the above mentioned cases recurrent nasal polyps requiring long-term, or permanent corticosteroid treatment are typical (Cepero et al., 1987; Szczeklik et al., 2000). Although the nasal polyps are often associated with other diseases, it is not probable that they are causative manifestation of those diseases.

1.2.6. Environmental factors

Although genetic factors influence development of NP, it is nowadays clear that this is not the most critical component in the etipathogenesis of the disease. Such a factor might come from the influence of the environment. Several environmental entities that could in combination with not very strong, but existing predisposing genetic factors, lead to the development of this frequent recurrent affection of nasal mucosa were investigated.

Environmental factors as food allergens, pollutants, inhalants and chronic viral, bacterial or fungal infection were suggested as possible causative agents in nasal polyposis. It has been proved in rodents that a control over a number of infectious pathogens (including viruses, bacterial, protozoan and mycotic infection) is directly dependent on

iNOS with iNOS induced NO expression having a direct antimicrobial effect (Fang, 1997). In this respect, increased expression of iNOS positive cells in nasal polyps compared to healthy nasal mucosa then indicates an environmental impact (Fundová et al., 2008). Role of infectious stimuli is considered important, especially by bacterial strains *Streptococcus pneumoniae, Staphylococcus aureus* or *Bacteroides fragilis* (usual pathogens in sinusitis) or *Pseudomonas aeruginosa,* often found in cystic fibrosis (Norlander et al., 1993). Recently, there has been a discussions about the effect of superantigens, derived especially from *Staphylococcus aureus,* on IgE secretion by B cells, as well as a direct effect of superantigens on characteristic cells of the inflammatory infiltrate in NP, i.e eosinophils (Bachert et al., 2008). The superantigens are involved in the modulation and aggravation of the airway inflammation (Huvenne et al., 2013). In the end of 20th century a group of Mayo researchers coined a terms "allergic fungal rhinosinusitis" (Ponikau et al., 1999) later changed to "eosinophilic fungal rhinosinusitis" as they were able to detect fungi in 96% of patients with CRS. Fungi have been suggested to play an important role in the pathogenesis of CRS (Chakrabarti et al., 2009). However, after an absence of convincing immunological data or evidence for clinical improvement of CRS upon therapy with antifungal agents the hypothesis that fungi play a role in a majority of the cases of CRS was rejected (Fokkens et al., 2012).

However, to these days no individual (nor a group of related) agents in NP were identified, thus a question arises, if such a specific etiologic entity does exist. Many of the environmental causative noxa were rejected in pathogenesis of NP and none of them was proven as critically important. Although this is partly also due to a non-existence of a well-established animal model for this disease. Thus, NP and CRS are at present considered environmentally driven multifactorial diseases, developing in genetically predisposed individuals. One of the less explored and recent topics in pathogenesis of NP is the role of physiological nasal microbiom and its interplay with physiology of nasal mucosa in subjects with nasal polyps (see next chapter).

1.2.7. Microbiom of the upper respiratory tract

The mucosal surfaces of the body of vertebrates are colonized by vast numbers of microorganisms, commonly referred as microbiota. (Kamada et al., 2013). Bacteria are present at anatomical locations that provide suitable conditions for their growth and proliferation. The upper airways, particularly the nasopharynx, harbor bacteria, as do some mucosal surfaces of the genital tract, although the greatest number of bacterial cells is

found in the digestive tract. Commensal bacteria are indispensable for the normal function of the mammalian organism. Most of the commensal bacteria are symbiotic; however, after translocation through the mucosa or under specific conditions, such as immunodeficiency, commensal bacteria could cause pathology (Tlaskalová-Hogenová et al., 2011). First, the microbiota of the large intestine was widely studied, later the interest moved also to different locations such as mucosal surfaces of the upper respiratory tract. In the project called "Human Microbiome" that was established to investigate the flora in healthy volunteers and their relationship to human health and disease, one of the emphasized body sites was nose/lung site (Human microbiome project, 2012).

The upper respiratory tract is colonized with numerous bacteria, with 10 to 100 anaerobes for every aerobic bacterium. The most common aerobic bacteria are *Streptococcus, Haemophilus* and *Neisseria* species. The most common anaerobic bacteria are *Peptostreptococcus, Veillonella, Actinomyces* and *Fusobacterium* species. The nares (nostrils) are always heavily colonized, predominantly with *Staphylococcus epidermidis* and *Corynebacteria*. In about 20% of the general population the nose is colonized with *Staphylococcus aureus,* thus nose being the main carrier site of this important pathogen (Todar, 2014). There is also evidence showing that, in the respiratory system, composition of airway microbiota varies between healthy people and people with diseases such as asthma (Goldstein-Daruech et al., 2011; Huang and Lynch 2011; Huang, 2013) and cystic fibrosis (Lynch and Bruce, 2013). The importance of intact commensal microbiota of the respiratory system was also demonstrated in viral infections, with the commensal microbiota composition critically regulating host immune response following influenza virus infection (Ichinohe et al., 2011). There were speculations that reduced contact of people with natural environmental features and biodiversity may adversely affect the human commensal microbiota and its immunomodulatory capacity. Some studies have shown a close relationship between CRS and *Staphylococcus aureus*, anaerobes and so on in the nasal cavity or paranasal sinuses, although the relationship between CRS and microorganisms in the gut has not been demonstrated (Suzaki et al., 2013). At present, studies dealing with the pathophysiology of upper airway diseases are necessary to establish the relationship between the microbiome and inflammatory patterns to find their clinical reflections and also their possible causal relationships (Chalermwatanachai et al., 2015).

1.2.8. Chemokines and cytokines

For nasal polyps is, apart from of the increased infiltration of inflammatory cells, also typical an elevated production of a range of inflammatory cytokines and chemokines that could by regulation of migration, survival and activation of the cells of inflammatory infiltrate substantially contribute to development and growth of NP and maintaining of the chronic inflammation in nasal mucosa. The eosinophil infiltration into the nasal mucosa is by a great deal modulated by GM-CSF from epithelial cells of nasal mucosa and secondly epithelial cells from NP (Xaubet et al., 1994). Chemokines as RANTES and eotaxin, that elevated in nasal polyps are also distinct chemoattractant of eosinophils (Nonaka et al., 1999; Allen et al., 1998). Although the GM-CSF intself can't directly influence adhesion and degranulation of eosinophils and ROS production, in the presence of cofactors (VCAM-1, ICAM-1) activation of eosinophils may be increased by GM-CSF (Shin et al., 2003). In addition, TNF-α and IL-4/IL-13 could elevate expression of VCAM-1 (vascular cell adhesion molecule-1) and thus facilitate migration of eosinophils in the tissue (Pawakar, 2003). In nasal polyps patients outside China, CRSwNP is an eosinophilic disease characterized by Th2 cytokines such as IL-5, eotaxin and IL-13 (Zhang et al., 2008). The IL-5 has been shown to be a defining cytokine in CRSwNP. Thus, IL-5 that is produced by activated T-lymphocytes and eosinophils fundamentally prolongs survival of the eosinophils by inhibition of apoptosis (Bachert et al., 1997; Simon et al., 1997). Cytokine IL-5 in nasal polyps is also highly correlated with other Th2 cytokines: eotaxin, MCP-1, MCP-4, TARC, IL-4 and IL-13 (Håkanson et al., 2015). In addition, eotaxin could also act locally within the nasal polyps and increase the tissue damage (Pawankar, 2003). It has been documented that the influx of either neutrophils, monocytes, NK cells, dendritic cells, mast cells, or T lymphocytes to the site of mucosal injury and into the inflammatory infiltrate is regulated by chemokines released from respiratory epithelial cells in response to pro-inflammatory cytokines (Brabcova et al., 2014). In addition, there is an elevated expression of IL-8 that chemotacticly attracts neutrophils. Next, proinflammatory cytokines such as IL-1β and TNF-α, that increase ICAM-1 expression on epithelial cells, could cause migration of T cells and neutrophils in nasal polyps. Finally, an engagement of P- and L-selectins in mediating binding of T cells in the mucosa of nasal polyposis has also been documented (Symon et al., 1999).

1.2.9. Mucosal immune system and epithelial cells

Mucosal immune system plays a principal role in the interaction of organism with an environment. Mucosa is the main mucosal site for tolerance induction to soluble antigens (Weiner, 1997). Interactions between segments β2 and β1 of integrins and their ligands very probably play an important role in mucosal immune response of upper respiratory tract (Demoly et al., 1998; McNulty et al., 1999). The mucosal barrier or immune exclusion is maintained by production of great amount of secretory IgA (sIgA). (Mestecky and McGhee, 1987). Commensals play an important role in induction and production of sIgA as well as in its specificities (Fagarasan, 2006). Pertinent changes of sIgA spectrum specificity (repertoire) in diseases affecting mucosal compartments have not yet been described. Commensals colonizing mucosa are in symbiotic coexistence with the host and belong to complex innate mechanisms protecting the organism against the pathogens. Bacterial populations create complex ecosystems. If their composition is optimal, the commensals prevent adhesion and reproduction of pathogens (Tlaskalova et al., 2002, 2004). Commensals represent also an important factor influencing development of mucosal immune mechanisms including non-responsiveness or "tolerance" of immune system to foreign environmental antigens not causing danger to the organism (Tlaskalova et al., 2004; Cebra, 1999).

A defect in epithelial cell homeostasis could represent and important factor in etiopathogenesis of NP. Epithelial cells of nasal mucosa are permanently exposed to many environmental factors, and comprise thus an important part of the mucosal immune system, as they e.g. produce broad spectrum of cytokines and chemokines (Stadnyk, 1993), express MHC class II glycoproteins (basolateral expression) as well as non-classical MHC class I glycoproteins (CD1d, TLA), and could serve as antigen-presenting cells for intra-epithelial lymphocytes (IEL) (Bleicher et al., 1990; Tlaskalova et al., 2004). IEL represent a unique lymphoid cell population as they develop outside thymus and often express organ specific γδ T cell receptor and CD8 αα homodimer. IEL play an important role in maintaining peripheral tolerance (Locke et al., 2006) as well as in regulation of growth of epithelial cells by producing keratocyte growth factor (KGF), that together with other growth factors (epidermal growth factor [EGF], transforming growth factor β [TGF-β], and insulin-like growth factors [IGFs] ensures the integrity and homeostasis of mucosal epithelium (Boismenu et al., 1996; Dignass, 2001). We have shown an increased number of insulin-like growth factor receptor-1 (IGF-1R) expressing cells in nasal polyps compared with non-

affected nasal mucosa (Fundova et al., 2008). IGF-1R prolongs a cellular life span and an increased expression of IGF-1R could contribute to mucosal hypertrophy in NP. Increased proliferation of epithelial cells was described in NP (Coste et al., 1996).

1.3. Type 1 diabates mellitus

1.3.1. Introduction

Insulin-dependent diabetes mellitus or type 1 diabetes (T1D) is an autoimmune disease, the result of a leukocyte attack on the beta cells of the islets of Langerhans within the pancreas. Mononuclear cells infiltrate the islets and cause insulitis leading to progressive and selective destruction of beta cells (Wang al., 1996). Although this disease is an ancient one, and one that is quite prevalent, its pathogenesis remains unclear. We do not know what triggers it, have only a sketchy knowledge of the sequence of events unfolding during its progression, and have very little understanding of the genetic and environmental factors which influence its course.

Type 1. diabetes occurs during childhood and adolescence, although not exclusively. Nevertheless, there is a worrying evidence for decreasing age of onset of T1D (Karvonen et al., 1999; Onkamo et al., 1999). In addition, th incidence of type 1 diabetes mellitus has been rapidly increasing during the past decades, preferentially in developed countries (Onkamo et al., 1999; Laakso et al., 1991). In humans, the autoimmune process is prolonged as clinical onset of the disease does not occur until approximately 80% or more beta cells are destroyed, leaving a time window of opportunity for therapeutical or preventive intervention in prediabetic individuals.

It has been documented that T cells play instrumental role in the development of human T1D, not only according to the mononuclear infiltrate present within the islets of Langerhans with domination of CD8^{+} T cells (Gepts, 1965; Itoh et al., 1993), but also due to the effect of immunosuppressive therapy in T1D and a presence of autoreactive T cell clones in human patients (Roep et al., 1999). Several autoantiboies were characterized in T1D, first antibodies binding to beta cells - ICA (islet cell antigens antibodies) (Bottazzo et al., 1974) and were also used for firs predictions of progression of the disease (Bonifacio et al., 1990). Later these autoantibodies were better indentified as anti-insulin (Palmer et al., 1983), anti-GAD65 (Baekkeskov et al., 1990), anti-tyrosin

phosphatase IA-2 (Notkins et al., 1996), as well as other were described e.g. anti-ganglioside GM2-1 (Dotta et al., 1992), and anti-sulphatide Abs (Buschard et al., 1993). Although autoantibodies do not play a major role in pathogenesis of the disease, they are used for assessing increased risk for the development of diabetes. Thus, detection of two or more antibodies confers increased risk to progress to the onset of the disease (Bingley, 1996). Anti-insulin and to a lesser extend anti-IA-2 antibodies may have better predictive value than anti-GAD65 Abs (Harrsion, 2001).

1.3.2. Animal models of type 1 diabetes

The non-obese diabetic (NOD) strain of mouse and Bio-breeding (BB) rats represent the two widely used rodent models developing spontaneously T1D. The BB rats were generated from a colony of Wistar rats, in which a few animals spontaneously developed diabetes (Nakhooda et al., 1977). Diabetes in BB rats is a result of auoimmune insulitis and apart of it BB rats also exhibit thyroiditis (Sternthal et al., 1981). They have earlier and faster onset of the disease than NOD mice. In the last decades, however, the number of studies carried out on BB rats declined, probably also due to practical obstacles – animal facilities, antibodies etc. and this fact has unfortunately narrowed our view on T1D.

The NOD mouse is at present the best established model of autoimmune type 1 diabetes. It is inbred and the NOD mice develop a disease very similar, by most evaluable criteria, to human T1D. In addition to diabetes, NOD mice present thyroiditis (Bernard et al., 1992), sialitis (Hu et al., 1992), autoimmune peripheral polyneuropathy, a systemic lupus erythematosus–like disease that develops if mice are exposed to killed mycobacterium (Silveira and Baxter, 2001), prostatitis (in male mice) and late in life, autoimmune hemolytic anemia (Baxter and Mandel, 1991). Interestingly, these various autoimmune syndromes are not always found in every animal. Recent data from Bach and colleagues (Alynakian et al., 2003) and others suggest that the various diseases may be individually regulated, and they may have unique antigen specificities. Finally, the NOD mouse strain is particularly susceptible to certain experimentally induced autoimmune diseases such as experimental autoimmune encephalomyelitis (Girvin et al., 2000). Thus, like some humans with T1D, the NOD mouse combines an overall genetic propensity for multi-organ autoimmunity with specific targets not limited to organs of the endocrine system. (Anderson and Bluestone, 2005).

NOD mice were originally developed by Makino and colleagues in Japan during the selection of a cataract-prone strain derived from the outbred Jcl:ICR line of mice (Makino et al., 1980). The incidence of spontaneous diabetes in the NOD mouse is 60% to 80% in females and 20% to 30% in males (Yoshida and Kikutani, 2000). Interestingly, the incidence of disease depends on the sanitary conditions in which mice are housed. Mice bred in a Specific Pathogen Free (SPF) environment are more prone to develop diabetes than mice bred in a conventional animal facility. When contaminated by different bacteria, viruses or parasites, SPF NOD mice do not develop T1D (Sadelain et al., 1990; Cooke at al., 1999; Oldstone, 1990). A germ-free (GF) or axenic status established under stringent conditions (i.e. mice born by caesarean section and housed in isolators) increases T1D incidence (Yurkovetskiy et al., 2013; King and Sarvetnick, 2011; Alam et al., 2011), the increase being more evident in GF males than females (Yurkovetskiy et al., 2013; Markle et al., 2013). Also, a directed even partial ablation of the gut microbiota, as induced by vancomycin, significantly increases type 1 diabetes incidence in male NOD mice (Candon et al., 2015). Diabetes onset typically occurs at 12 to 14 weeks of age in female mice and slightly later in male mice. Histological studies have shown that few immune cell infiltrates are noted in islets until approximately 3 to 4 weeks of age, when both male and female mice begin to demonstrate leukocytic aggregates and mononuclear infiltrates that surround the islet (peri-insulitis). These lymphocytic infiltrates progress and invade the islets (insulitis) over the subsequent few weeks, such that most mice demonstrate severe insulitis by 10 weeks of age.

The dominant initial population consists of $CD4^+$ T cells followed by infiltration of $CD8^+$ T cells, macrophages and B cells in smaller numbers (Zhang et al., 2008). Diabetes induction is possible with many of these clones in the absence of B cell help or other T cell lines. NOD disease is primarily dependent on $CD4^+$ and $CD8^+$ T cells (Wicker et al., 1986; Bendelac at al., 1987). Evidence for this includes the ability to transfer disease with purified $CD4^+$ and $CD8^+$ T cells from NOD donors, the ability of individual T cell clones (both class I and class II restricted) derived from NOD islets to passively transfer disease (Haskins and Wegmann, 1996; Wong et al., 1996), and the fact that T cell modulating therapies inhibit disease incidence (Shizuru et al, 1988; Chatenoud et al, 1994; Wang et al., 1996; Kurasawa et al., 1993). Whereas diabetes can be transferred from affected animals by passive transfer of splenocytes, it cannot be transferred by autoantibodies from new onset diabetic donors, although B cells also participate in the development of the disease (Serreze et al., 1998). The finding that the reduced incidence in male mice occurs in spite of similar

levels of early insulitis suggests that late regulatory events control disease progression. Thus, the autoimmune process in the pancreas of NOD mice includes two checkpoints: checkpoint 1, or insulitis, which is completely penetrant; and checkpoint 2, or overt diabetes, which is not completely penetrant (Andre et al., 1996). While it is accepted that T1D in NOD is primarily T cell-mediated, NOD mice also develop autoantibodies to insulin first detectable at 6 weeks of age and peaking between 8 and 16 weeks. These antibodies themselves do not cause diabetes, but they contribute significantly to the rate of disease progression (Greeley et al., 2002; Kagohashi et al., 2005; van Belle et al., 2009).

Other animal models include accelerated diabetes in NOD by cyclophosphamide via its effect on Tregs (Yasunami and Bach, 1988). Cyclophosphamide selectively eliminates Tregs and also causes rapid re-infiltration of islets with activated macrophages and influx of $CD4^+$ T cells (Kay, 1991). Streptozotocin can induce diabetes at high dose by a toxic effect, whereas low dose streptozocin induces autoimmune diabetes in many mouse strains but not the NOD mouse (Kay et al., 1991). Last but not least, there is a adoptive transfer model of diabetes, that allows to test regulatory capacity of various experimentally induced cell subsets. It includes transfer of diabetogenic splenocytes from recently diabetic NOD donors to NOD-irradiated or NOD-SCID recipients (Christianson et al., 1993). Regulatory or inhibitory properties of various cell subsets could be tested in a co-transfer setting.

1.3.3. Genetic factors

Genetic factors contribute to the development of T1D, although their role has been for long period of time overestimated (Bach, 1994). HLA genes represent the major predisposition to the disease (Todd and Farrall, 1996). Increased risk is associated with Class II HLA DQ and DR genes. While HLA-DQ alleles have a dominant effect, HLA-DR alleles display modulating influences (She, 1996). The highest risk is linked to DQ2/DQ8 phenotype which is found in more than 30% of T1D patients. Within MHC Calls I region a polymorphism in TNF-α was suspected and later polymorphism in MIC (MHC class I chain-related) gene was mapped in the proximity of the TNF-α gene (Gambelunghe et al., 2000).

Other risk locus was localized on chromosome 11p15, close to the insulin gene (Bell et al., 1984) possibly influencing insulin expression in the thymus and thus central tolerance mechanisms. In NOD mice, a region of IL-2 gene has been also identified, possibly explaining lower IL-2 responses found in NOD mice (Lyons et al., 2000). All

together more than 40 possible loci have been reported in type 1 diabetes, the HLA loci having a large affect, but still only conferring predisposition to the disease development (Morran et al., 2015).

1.3.4. Environmental factors

Environmental factors play an important role in the recent increase of type 1 diabetes (T1D), especially in developed countries (Graves and Eisenbarth, 1999; Rewers and Ludvigsson, 2016). There is a remarkable geographical difference (Diabetes Epidemiology Group, 1988) in the prevalence of the disease. Thus, the highest incidence in Sardinia and Finland reaches over 36 cases/100,000 per year, whereas in China incidence is a low of 0.1/100,000 per year as reported by the WHO DiaMond project. The geographical variation cannot be explained solely on genetic differences as migrants adopt the incidence of their host country (Patrick et al., 1989; Rewers et al., 1987).

The fact that more than 50% of identical twins are discordant for T1D also point to the importance of environmental factors, in fact environmental factors are estimated to contribute by more than 70% to the development of T1D (Harrison and Honeyman, 1999). Furthermore, during a relatively short period of a few decades the incidence substantially increased, especially in the developed countries (Laakso et al., 1991). Thus genetic susceptibility is necessary for the development of T1D, but the disease incidence is driven by environmental factors or rather recent environmental changes. The exact environmental factors and their mechanisms are not known but dietary factors (wheat proteins), infections, lack of breastfeeding, early immune stimulation versus clean "hygienic" environment, gut microbiom are being studied in this respect.

In the NOD mouse, under strict SPF conditions, diabetes occurs earlier and the incidence is greater (Pozzilli et al., 1993). Perhaps stimulation of the intestinal immune system is thought to modulate the development of autoimmunity in these genetically-susceptible mice. Feeding of bacteria, such as Lactobacillus casei (Matsuzaki et al., 1997), helminth infection (Cooke et al., 1999), mycobacterium infection (Martins and Aguas, 1999) all decrease spontaneous diabetes. Stress (handling at a young age reduces incidence), has also been demonstrated to reduce diabetes incidence in NOD mice. In accord with the hygienic hypothesis, until relatively recently humans were exposed to a greater variety of infectious agents. Thus the current freedom from infection (due to the vaccines, abuse of antibiotics and cleaner living environments) may cause clinical onset of

T1D to occur in many more predisposed individuals then before. This hypothesis is supported by fact that children attending kindergarten are at lower risk of developing T1D (Rami et al., 1999). Treatment with antibiotics also reduces diabetes incidence in both NOD mice and BB rats (Hansen et al., 2012; Buschard et al., 1992). Similarly, vancomycin, significantly increased type 1 diabetes incidence in male NOD mice (Candon et al., 2015). A protective role of gut SPF microflora is also supported by the finding that rederivation of an NOD colony highly increases its diabetes incidence (Wilberz et al., 1991). In addition, germ-free conditions increases diabetes incidence in both females and males and also lead to rapid onset of the disease (Funda et al., 2007; Yurkovetskiy et al., 2013; King and Sarvetnick, 2011; Alam et al., 2011). In addition, there could be interplay between diabetes preventive diets and gut microflora. While diabetes preventive effect of some diets is microflora-dependent, the effect of the gluten-free Altromin diet is microflora-independent (Funda et al., 2007).

In both NOD mice and BB rats, type 1 diabetes is a diet-influenced disease. Role of cow`s milk proteins in T1D was intensively studied for certain period and was a rather controversial topic (reviewed in Harrison and Honeyman, 1999). It has been suggested that early introduction of cows' milk to children or a lack of breast-feeding may trigger T1D (Borch-Johnsen et al., 1984). However, a study in high-risk infants in Denver (Norris et al., 1996) analyzed the duration of breastfeeding and the time of introduction of cow's milk and dairy products and found no association between infant feeding and the development of islet autoimmunity. The development of spontaneous diabetes in either NOD mice or BB rats is not dependent on exposure to cows' milk proteins (Paxson et al., 1997). Thus, this evidence does not support a pathogenic role for cows' milk. On the other hand it is difficult to separate early exposure to bovine antigens from a lack of breast milk. Breast milk contains a lot of growth factors and cytokines, many of which have a role in the maturation of intestinal mucosal tissues (Xanthou et al., 1995). Thus, early withdrawal of breast milk could impair mucosa-mediated tolerance to dietary antigens (including islet autoantigens such as insulin), (Harrison and Honeyman, 1999).

Diabetes incidence in NOD mice and BB rats is strongly influenced by dietary wheat proteins (Scott, 1996; Coleman et al., 1990). It is well documented that the diabetes-promoting dietary components are not carbohydrates but come from the plant protein fraction of semi-purified open formula animal diets (Hoorfar et al., 1991; Hoorfar et al., 1993). In BB rats, Scott (Scott, 1996) has tested delayed introduction of a diabetogenic diet (50 or 100 days) and showed that exposure to food at age 50 to 100 days (corresponding to

the period of early puberty to late adolescence in humans) is critical for the dietary modulation of diabetes development. Several studies documented that gluten-free diet highly decreases diabetes incidence in NOD mice (Funda et al., 1999; Schmid et al., 2004). The early introduction of a gluten-free diet to NOD mice decreased the incidence of diabetes from 64% to 15% and significantly delayed the onset of diabetes (Funda et al., 1999). Gluten, a major protein in wheat flour, when added to a semi-synthetic diet at weaning, it increased the incidence of diabetes from 15% to 35% in BB rats (Elliott and Martin, 1984). However, the effect of diets is even more complex. It has been shown that the diabetes-preventive gluten-free diet tested in NOD mice modifies the composition of gut microflora (Hansen et al., 2006). Diabetes incidence in NOD mice is also highly influenced by intestinal microbiota (see above), nevertheless effect of some diabetes-preventive diets is microflora-dependent, while effect of the gluten-free diet in NOD mice is microflora independent (Funda et al., 2007). A human prospective trial with at risk first-degree T1D relatives kept on gluten-free diet has been carried out, documenting a beneficial effect on preservation of beta cell function (Pastore et al., 2003). There is also a case report of a newly diagnosed type 1 diabetic on gluten-free diet without need of insulin therapy (Sildorf et al., 2012).

The mechanism how diet modification influence diabetes incidence is not known. A shift from a Th1 to Th2 cytokine pattern has been described in the islet infiltrate of BB rats fed a hydrolyzed casein diet (Flohe et al., 2003). We have recently documented that dietary gluten alters cytokine profile of T cells to more pro-inflammatory one and it is associated with increased proportion of Th17 cells but decreased proportion of mucosal γδ T cells (Antvorskov et al., 2012, 2013). Gluten displays lectin like properties (Kottgen et al., 1983) and similarly to LPS directly stimulates innate immune responses through activation of NF-kappaB (Jelinkova et al., 2004) as well as influences maturation of dendritic cells (Palova-Jelinkova et al., 2005).

Direct infection of the pancreas may trigger autoimmune responses, however, evidence of local viral infection is sparse due to the difficulty in isolating RNA from the pancreas. On the other hand, enteric viruses localized to the intestine could indirectly trigger autoimmune processes by affecting mucosal permeability or altering the balance of mucosal immune system. Entroviruses such as coxsackie B4 (Andreoletti et al., 1998), echovirus (Sadeharju et al., 2001) and also rotavirus (Honeyman et al., 2000) have been implicated in T1D. Sequence similarities have been found between several enteroviral

antigens and the immunodominant epitopes of autoantigens in T1D, including similarities between coxsackie and GAD as well as rotavirus and GAD65 and IA-2 (Honeyman et al., 1998; Atkinson et al., 1994). However, direct evidence for molecular mimicry as a mechanism initiating β-cell autoimmunity is lacking. Nevertheless, there is a significant correlation between rotavirus infections and induction of autoantibodies in at-risk hildren of type 1 diabetes (Honeyman et al., 2000). On the other hand, there are several viruses (e.g. mouse hepatitis virus, MHV), that lower diabetes incidence in NOD mice (Bach, 1994).

1.3.5. Type 1 diabetes and celiac disease

Celiac disease (CD) is an inflammatory T cell-mediated disorder of the small intestine and develops because of ingested gluten fraction of wheat or the homologous proteins from barley and rye, in genetically predisposed individuals. An increased number of intraepithelial lymphocytes and lamina propria cells and their activation, followed by villous atrophy and crypt hyperplasia, characterize CD. Both innate and adaptive immune responses contribute to the onset of mucosal inflammation in CD patients (Stepniak and Koning, 2006). Fragments of gliadin – a major group of proteins in gluten – cross the epithelium, and are presented by antigen presenting cells to the HLA-DQ2 or HLA-DQ8-restricted CD4+ α/β T lymphocytes present in jejunal mucosa (Sollid, 2002). The disease, which can be treated effectively by a gluten-exclusion diet, is prevalent among Caucasians (1 in 200 individuals), but only 20–50% of those individuals that are affected have symptoms. Celiac disease is an acquired disorder; it can be diagnosed in early childhood with classical symptoms, such as diarrhoea and malabsorption, but it can also be diagnosed later in life, and there is a wider spectrum of symptoms in adults than in children. Poor diet compliance and undiagnosed disease are associated with increased morbidity and mortality (Fasano and Catassi, 2001; Sollid, 2002; Lundin et al., 2015).

There is a higher association or T1D and celiac reported in different countries by several authors (reviewed in Cronin and Shanahan, 1997). The association was first accounted to the genetics – as both disease share the DR3 and DQ2 risk alleles. However, there is also an evidence for a functional role of gliadin in T1D. Thus in humans, patients diagnosed with both CD and T1D usually develop diabetes first and not vice versa - an indirect suggestion for an effect of gluten-free diet on the development of T1D (Cronin and Shanahan, 1997, Cosnes et al., 2008). Second, regions with a low diabetes incidence such as in Asia (Japan, Korea) also

have a much lower consumption of wheat flower proteins. Possible ethiological role of gliadin could be also implicated from the fact that patients with celiac disease have an earlier onset of T1D (Hansen et al., 2006) and there is also one report of enhanced T reactivity to gluten in newly diagnosed type 1 diabetics (Klemetti et al., 1998). Early introduction of dietary gluten was reported to increase the risk of developing islet autoantibodies in children (Norris et al., 2003). Gluten-free diet also induces changes in the gut microbiota of NOD mice (Hansen et al., 2006) The etiological role of gliadin in T1D is supported by a study documenting that while sensitization with gliadin induces only moderate enteropathy in humanized NOD-DQ8 mice, when combined with partial antibody depletion of Foxp3 Tregs, it led to development of insulitis (Galipeau et al., 2011). This is a rare example of induction of insulitis, as most manipulations in animal models lead to T1D prevention. Thus, although indirect, there is an evidence for a functional association of the CD and T1D.

2. SIGNIFICANCE, OUTLINE & AIMS OF THE THESIS

Mucosal immune system represents the major compartment of the immune system that is also responsible for its interactions with the outside world. Mucosal surfaces serve as barrier against but also as an interface to the outer environment. Mucosal surfaces are the site of specific proportions of various immune responses including innate immune responses, tolerance induction as well as specific migration and cell/antigen trafficking within this compartment. Thus, mucosal immune system is instrumental as the first line of defense against danger signals form the outer environment as well as for maintaining immune homeostasis with non-danger events such as many inhaled agents, dietary components and commensal microbes inhabiting mucosal surfaces (nose, oral cavity, gut etc.). Mucosal immune system has been long understudied, perhaps also due to the limited accessibility, however, it is now for many years established that local immune mechanisms and tissue specific immune responses are important in pathogenesis of organ specific diseases.

The respiratory tract is constantly exposed to number of inhaled antigens including allergens, viral, fungal and bacterial components. As the upper part of respiratory tract is the first place of contact, the load of inhaled antigens is higher than in the lower part. However, as the asthma research is quite intense, much less attention has been given to the inflammatory disease of the nose. The nasal polyps (NP) represent a chronic disorder of nasal mucosa and nasal sinuses mucosa. Despite its high frequency and their complications, there is no proper understanding of the etiology and to a great degree also pathogenesis of the disease.

The frequency of autoimmune diseases is constantly rising in the population, especially in the developed world. This is also the case for autoimmune type 1 diabetes. As the genetic factors could not explain the substantial population increase of T1D in the last decades, the research attention focuses more and more on environmental factors such infections and diet, which could influence the incidence and penetrance of the disease.

In this thesis I studied the mucosal immune system in a local mucosal affection - in nasal polyposis, but also in an organ specific autoimmune disease - type 1 diabetes, in which mucosal delivery of not only autoantigens, but also environmental entities (dietary components, probiotics) should be "re-searched" for their use in secondary prevention of

the disease. Mucosal immune system in diseases – especially mucosal immune system of the upper respiratory tract and of the gut is subject of this thesis.

This thesis consists of two parts that also reflect my involvement in both experimental immunology research as well my clinical ENT practice. They are interconnected not only by my interests but also by the common mucosal immune system phenomenon, that e.g. allows for intranasal vaccination in type 1 diabetes prevention.

Thus, the first, more clinically focused, part deals with the role of mucosal immunity of the upper respiratory tract in disease conditions - more specifically in nasal polyposis, a recurrent, chronic inflammatory condition, which pathogenesis and etiology are not well understood. We focused :

- on mechanism of the massive eosinophilic as well as neutrophilic infiltration in pathogenesis of NP by studying pattern of expression of CCR1 and CCR3 chemokine receptors in nasal polyps versus nasal mucosa.

- on the possible role of epithelial cell homeostasis and innate immune mechanisms in pathogenesis of NP by evaluating expression of insulin-like growth factor-1 receptor (IGF-1R) and iNOS in stroma and epithelium of NP compared to nasal mucosa.

The second part of this thesis deals with the role of mucosal immunity and environmental factors in autoimmune diseases - more specifically, in the spontaneous animal model (the NOD mouse) of type 1 diabetes. We investigated effects of environmental factors such as gluten-free diet and gliadin on the mucosal immune system and prevention of type 1 diabetes, and also addressed some innate mechanisms of gliadin signaling by:

- studying possible mechanisms by which gluten-free diet highly prevents type 1 diabetes in NOD mice; in particular its influence or regulatory T cells and Th17 cells in mucosal compared to systemic lymphoid compartments.

- further investigating the diabetes-protective effect of gluten-free diet by assessing the balance of pro-inflammatory and anti-inflammatory cytokines in T cells in the mucosal versus systemic lymphoid compartments.

- mapping innate signaling pathways of pepsin-digest of gliadin (such as TLR2/4/MyD88/TRIF/MAPK/NF-κB and a NLRP3 inflammasome activation), also in relation to celiac disease.

- testing intranasal vaccination strategy with gliadin, an environmental agents, that may have etiological role in type1 diabetes, for both prevention or even early cure of type 1 diabetes. Describing induction of Tregs and γδ T cells and their cytokine profiles in relation to this immunointervention strategy.

In addition, two reviews that cover the two parts described above are presented in this thesis.

3. RESULTS AND DISCUSSIONS

3.1. Clinically focused studies: mucosal immunity of the upper respiratory tract in nasal polyposis and CRC.

3.1.1. Publication I.

Fundová P, Funda DP, Kovář D, Holý R, Navara M, Tlaskalová-Hogenová H. Increased expression of chemokine receptors CCR1 and CCR3 in nasal polyps: molecular basis for recruitment of the granulocyte infiltrate. *Folia Microbiol* **(Praha), 2013, 58(3):219-24**. IF (2015) = 1.335

Nasal polyposis (and chronic rhinosinusitis, CRS) is a recurrent and most likely multifactorial chronic disease of the upper respiratory tract with still unclear etiology and pathogenesis (Fokkens et al., 2005; Fokkens et al., 2012). This is perhaps also due to a non-existence of a good animal model for this rather frequent nasal affection within ENT practice. Nevertheless, the massive eosinophil, and to a lesser extend neutrophil infiltration typical for most common type of nasal polyposis represent together with various proinflammatory and allergy related cytokines and chemokines an important phenomenon for experimental studies (Pawankar, 2003; Stoop et al., 1993). Using human bioptic material several studies reported increased expression of cytokines and chemokines such as e.g. TNF-α, IL-4, IL-5, IL-8, IL-13, RANTES, eotaxin as well as IL-1β, TNF-α, TGF-β and others in NP and CRS (Bachert et al., 2002; Shin et al. 2003; Pawankar, 2003; Bernstein et al., 2004). With respect to the high accumulation of eosinophils and neutrophils in NP, chemokine receptors CCR1 and CCR3 represent molecular structures involved in recruitment of this inflammatory infiltrate.

The aim of this study was to investigate the expression of chemokine receptors CCR1 and CCR3 in nasal polyps compared to nasal mucosa. Both computer image analysis and fluorescence microscopy with calibrated eyepiece graticule were used to asses the differences in expression. All samples were collected form patients with no sign of asthma and allergies. We found increased number of cells expressing CCR1 and CCR3 in the stroma of nasal polyposis. Next, we also examined the pattern of CCR1 and CCR3

expression and documented increased positivity of CCR3 within the epithelial compartment of NP.

In conclusion, our data document molecular basis for recruitment of the neutrophil and eosinophil infiltrate in NP. Increased or dysbalanced production of chemokines and cytokines may facilitate accumulation of immune cells expressing corresponding receptors CCR1 and CCR3. Because eosinophils and neutrophils form the predominant cellular component in NP, we think they may also represent important targets for novel therapies. Their active role in pathogenesis of NP should be further investigated as novel mechanisms such us extracellular DNA traps were described with respect to these granulocytes (Goldmann and Medinna, 2013). They bring new light on their pathological and possibly also physiological roles (e.g. in sterile inflammation, prolonged immunoregulation) in the mucosal immune responses of the nose.

Increased expression of chemokine receptors CCR1 and CCR3 in nasal polyps: molecular basis for recruitment of the granulocyte infiltrate

P. Fundová · D. P. Funda · D. Kovář · R. Holý · M. Navara · H. Tlaskalová-Hogenová

Abstract Inflammatory processes play an important role in the development of nasal polyps (NP), but the etiology and, to a high degree also, the pathogenesis of NP are not fully understood. The role of several cytokines and chemokines such as eotaxins, IL-4, IL-5, IL-6, IL-8, and RANTES has been reported in NP. Herewith, we investigated the expression and pattern of distribution of chemokine receptors CCR1 and CCR3 in nasal polyps. Immunohistochemical detection was carried out in frozen sections of biopsies from 22 NP and 18 nasal mucosa specimens in both the epithelial and stromal compartments. Fluorescence microscopy and computerized image analysis revealed a statistically significant increased number of CCR1 (45.2 ± 2.8 vs. 15.1 ± 1.9, $p < 0.001$)-positive as well as CCR3 (16.4 ± 1.4 vs. 9.7 ± 1.1, $p < 0.001$)-positive cells in the stroma of NP compared to nasal mucosa. In comparison to healthy nasal mucosa, increased positivity of CCR3 was detected in the epithelial compartment of NP. Our data suggest that increased expression of CCR1 and CCR3 chemokine receptors may, in accord with various chemokines, contribute to the pathogenesis of nasal polyposis by facilitating increased migration and prolonged accumulation of inflammatory cells, e.g., eosinophils, in the inflammatory infiltrate of NP.

Abbreviations

CCL	C-C chemokine ligands
CCR1	C-C chemokine receptor type 1
CCR3	C-C chemokine receptor type 3
CD	Cluster of differentiation
CY3	Indocarbocyanine
GM-CSF	Granulocyte/macrophage colony-stimulating factor
H&E	Hematoxylin & eosin
ICAM-1	Intercellular adhesion molecule 1
IgG	Immunoglobulin G
IL	Interleukin
MCP-3	Monocyte chemoattractant protein 3
MCP-4	Monocyte chemoattractant protein 4
MEC	Mucosae-associated epithelial chemokine
MIP-1 α	Macrophage inflammatory protein 1α
MIP-3	Macrophage inflammatory protein-3
NFκ-B	Nuclear factor-kappaB
NM	Nasal mucosa
NP	Nasal polyps
RANTES	Regulated on activation, normal T expressed and secreted protein
TBS	Tris-buffered saline
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1

P. Fundová (✉) · D. Kovář · R. Holý · M. Navara
ENT Department of the 3rd Faculty of Medicine, Charles University and the Central Military Hospital,
U vojenské nemocnice 1200,
169 02 Prague 6, Czech Republic
e-mail: pdfunda@seznam.cz

P. Fundová · D. P. Funda · H. Tlaskalová-Hogenová
Department of Immunology and Gnotobiology, Institute of Microbiology, v.v.i, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Introduction

Nasal polyps (NP) represent a frequent and recurrent chronic inflammatory disease of nasal and paranasal sinus mucosa. Histologically, NP are characterized by edema and loose connective tissue, hyperplasia within both stromal and epithelial tissue compartments, metaplasia of the surface epithelium, thickening of the basement membrane, and reduction of mucous glands and goblet cells. Nasal polyps are also characterized by a chronic inflammatory infiltrate that comprises first of all of eosinophils and also neutrophils,

mast cells, monocytes/macrophages, as well as T lymphocytes with increased proportion of activated and CD8^{+} T cells (reviewed in Pawankar 2003; Bachert et al. 2003; Fokkens et al. 2005).

Chronic inflammatory processes play an important role in development of NP, but both etiological factors as well as exact pathogenetic mechanisms of NP are still unclear.

Increased expression of several proinflammatory chemokines and cytokines such as eotaxin (CCL11), regulated on activation normal T expressed and secreted protein; RANTES (CCL5), IL-1β, IL-4, IL-5, IL-8, TNF-α but also TGF-β and granulocyte/macrophage colony-stimulating factor (GM-CSF) were reported in nasal polyps (Pawankar 2003; Bachert et al. 1997, 2002; Shin et al. 2003; Bernstein et al. 2004). The increased influx of eosinophils that form more than 80 % of infiltrating cells (Stoop et al. 1993) is facilitated by increased levels of eotaxin (CCL11), RANTES (Meyer et al. 2005; Nonaka et al. 1999; Allen et al. 1998), IL-5 (Bachert et al. 1997), and also GM-CSF (Xaubet et al. 1994). Similarly, increased levels of IL-1β, TNF-α, and IL-8 in NP may contribute to the influx of neutrophils (Pawankar 2003). The above-mentioned chemokines and cytokines can be produced by a variety of cell types, e.g., fibroblasts, mast cells, and T cells. Among them, epithelial cells represent a very potent source of various chemokines and cytokines such as IL-8, eotaxin, RANTES, TGF-β, GM-CSF, etc. (Stadnyk 2002; Shin et al. 2003).

CCR1 is a chemokine receptor, for which several ligands such as CCL5 (RANTES), CCL3 (macrophage inflammatory protein 1α, MIP-1 α), CCL7 (monocyte chemoattractant protein 3, MCP-3), CCL23 (macrophage inflammatory protein-3, MIP-3), and several other chemokine (C-C motif) ligands (CCLs) have been identified (Neote et al. 1993; Gao et al. 1993; Murphy 2002; Berahovich et al. 2005). This chemokine receptor is involved in the recruitment of inflammatory immune cells—above all neutrophils, monocytes/macrophages, and dendritic cells but also lymphocyte subsets (Murphy 2002).

C-C chemokine receptor 3 (CCR3), a key receptor for eosinophil migration, is apart from eosinophils expressed also on mast cells, basophils, and subset of T cells with Th2 cytokine profiles (Daugherty et al. 1996; Ponath et al. 1996; Sallusto et al. 1997). CCR3 was documented to bind all three eotaxins (CCL11, CCL24, and CCL26), RANTES (CCL5), MIP-1α (CCL3), MCP-4 (CCL13), and mucosae-associated epithelial chemokine MEC (CCL28) (Murphy 2002; Laing and Secombes 2004). The interplay of many various chemokines with the CCR1 and CCR3 receptors as well as the fact that CCR3 was shown to participate in eosinophil arrest and accumulation (Kitayama et al. 1998) points to their role in nasal polyposis.

The present study was designed according to a hypothesis that leukocytes may infiltrate and accumulate in nasal mucosa by mechanisms dependent on expression of chemokine receptors and that increased expression of chemokine receptors not only by infiltrating cells but also epithelial cells may play a role in the chronic pathogenesis of nasal polyposis. Thus, in this study, we determined the expression of CCR1 (CD191) and CCR3 (CD193) chemokine receptors in biopsies of nasal polyps compared to healthy nasal mucosa. By using immunohistochemical detection on frozen tissue sections and quantitative microscopic analyses, we determined the pattern of expression and quantified the number of positive cells.

Materials and methods

Subjects and specimens

Tissue samples of nasal polyps were obtained from 22 subjects with bilateral nasal polyps undergoing polypectomy for nasal obstruction. Control tissue samples of nasal mucosa were obtained from the same 12 subjects as well as from 6 subjects undergoing nasal surgery for the deviation of nasal septum. All of the subjects were non-asthmatic patients and did not receive any glucocorticoid treatment during a period of at least 12 weeks prior the biopsy. Informed consent for collecting biopsies for this study was obtained from all the patients, and the study protocol was approved by the Ethical Committee of the Central Military Hospital, Prague, Czech Republic.

Biopsies of about 4×4×2 mm were collected from the antero-inferior part of the inferior turbinate and from the most anterior polyp. Xylocaine aerosol (10 %) was used as local anesthetic. The biopsies were immediately embedded in optimal cutting temperature compound (Tissue-Tek, Miles Scientific, IN, USA), snap frozen in iso-pentane (BHD Laboratory Supplies, UK), and stored at −80 °C. Cryosections were cut serially at 5–7 μm, dried overnight at room temperature, fixed in acetone for 10 min, and used for immunohistochemistry.

Immunohistochemical staining

Nonspecific binding was blocked with normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) diluted 1:100 in TBS for 30 min at room temperature. Tissue sections were then incubated in a moist chamber with primary rabbit anti-human CCR1 and CCR3 antibodies (Abs) at room temperature for 60 min. Microscopic slides were then rinsed three times for 5 min in TBS. Rabbit anti-human peptide-affinity purified polyclonal antibodies to CCR1 and CCR3 were obtained from Imgenex (San Diego, CA, USA). The incubation with secondary multiple absorbed Cy3-conjugated goat anti-rabbit IgG F(ab′)$_2$ (Jackson Immunoresearch Lab., West Grove, PA, USA) was performed at room temperature in the

dark for 30 min. Slides were then quickly counterstained with hematoxylin and mounted in aqueous mounting medium (Faramount; DAKO, Denmark). Tissue sections with omitted primary antibodies served as negative controls.

Cell counting and computer image analysis

All tissue sections were coded and blindly examined using fluorescence microscopy in both epithelial and lamina propria compartments. Positive cells were counted under the Olympus AX70 Provis (Olympus, Tokyo, Japan) and Zeiss Axiovert 200M (Carl Zeiss, Jena, Germany) fluorescence microscopes at magnifications ×200 and ×400 using a calibrated eyepiece graticule as well as by computer image analysis (analySIS®3.0, Soft Imaging System GmbH, Germany). The magnification was matching that used with the calibrated eyepiece graticule. The number of positive cells has been counted in 40 randomly selected fields and in 20 pictures corresponding to the calibrated eyepiece graticule fields.

Statistical analysis

Data are given as mean ± standard error of mean (SEM), and the level of significance was assessed by unpaired two-tailed t test. Data were analyzed by GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

Distribution of the C-C chemokine receptor 1 (CCR1) and 3 (CCR3) positivity was investigated by counting with a calibrated eyepiece graticule in both the stromal (lamina propria) and epithelial compartments of 22 nasal polyp biopsies and of 18 nasal mucosa biopsies (Table 1). No differences in the pattern of distribution of CCR1 and CCR3 expressions

as well as numbers of positive cells were found between biopsies of healthy nasal mucosa from the inferior turbinate obtained from subjects with nasal polyposis ($n=12$) and subjects undergoing nasal surgery for the deviation of nasal septum ($n=6$). Thus, data representing nasal mucosa (as shown in Table 1) comprise biopsies of healthy nasal mucosa from both subgroups.

Expression of CCR1 in healthy nasal mucosa was confined to a small number of scattered, isolated CCR1-positive cells whereas remarkably increased number of CCR1-positive cells (45.2 ± 2.8 vs. 15.1 ± 1.9, $p<0.001$) was documented in the stroma of NP compared to nasal mucosa (Table 1; Fig. 1a, b). The CCR1 expression in NP was distributed within the entire lamina propria compartment with some positive cells accumulated along the basal membrane underneath the epithelial layer (Fig. 1a). Low levels of CCR1 positivity were detected in endothelial cells of both nasal mucosa and NP biopsies.

Detection of fluorescence positivity for the C-C chemokine receptor CCR3 showed an increased expression by epithelial cells of NP (Fig. 1c). Fluorescence microscopy and computer image analysis revealed that approx. up to 20 % of epithelial cells of NP displayed positivity for CCR3 in all biopsies of NP, while no or very low epithelial expression of CCR3 was observed within the epithelium of nasal mucosa biopsies (Fig. 1c, d; Table 1). Similarly, nasal polyps were characterized by statistically significant increase of solitary, CCR3-positive cells in the stroma of NP (16.4 ± 1.4 vs. 9.7 ± 1.1, $p<0.001$) compared to healthy nasal mucosa biopsies (Table 1; Fig. 1c, d). There was no detectable expression of CCR3 by endothelial cells of high endothelial venules within the lamina propria compartment. In conclusion, both increased CCR3 positivity of epithelial cells as well as significantly increased numbers of CCR3-positive stromal cells were found in NP compared to nasal mucosa.

Table 1 CCR1 and CCR3 positivity in nasal polyps compared to healthy nasal mucosa

Specimen		Nasal polyps ($n=22$)	Nasal mucosa ($n=18$)
CCR1	Epithelium	nps[a]	nps[a]
	Stroma	45.2±2.8	15.1±1.9[*]
CCR3	Epithelium	≤20 %	nps[a]
	Stroma	16.4±1.4	9.7±1.1[*]

Proportion of positive staining within the epithelium (in percent; *nps* no positive staining) and number of positive cells in the stroma (values are given as means ± SEM). Epithelial cell staining was assessed, and positive cells were calculated as described in the "Materials and methods"

[*] $p<0.001$ indicating statistical significance within the same tissue compartment

[a] No positive staining

Discussion

In this study, we describe the expression pattern of chemokine receptors CCR1 and CCR3 in both lamina propria and epithelial compartments of nasal polyps compared to physiological nasal mucosa (Fig. 1). Increased expression of CCR1 and CCR3 within the stroma of NP (Table 1, Fig.1) corresponds with the well documenting immuno-inflammatory infiltrate of NP (Pawankar 2003; Bachert et al. 2003; Sánchez-Segura et al. 1998; Bernstein et al. 2004). The accumulation of eosinophilic infiltrate as well as other leukocytes (e.g., neutrophils) in airway mucosa is facilitated by intercellular adhesion molecules (Symon et al. 1999) such as ICAM-1, ICAM-2, and VCAM-1 that display increased endothelial expression in nasal polyps (Tingsgaard et al. 1998, 1999) as well as by locally increased levels of

Fig. 1 Immunofluorescence detection of CCR1 and CCR3 positivity in nasal polyps and nasal mucosa. Photomicrographs of frozen sections of nasal polyps and healthy nasal mucosa biopsies stained for CCR1 (**a** and **b**, respectively) and CCR3 (**c** and **d**, respectively). Magnification, ×400
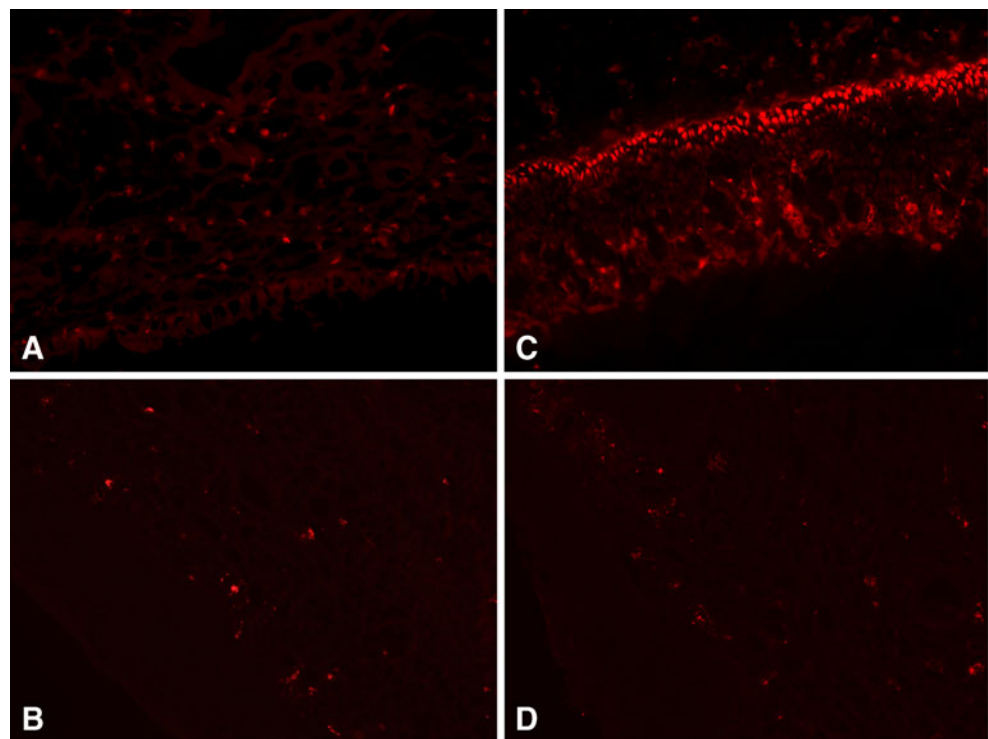
several chemokines and cytokines, e.g., IL-1β, IL-5, IL-6, IL-8, TNF-α, RANTES, and eotaxins (Pawankar 2003; Bachert et al. 1997; Meyer et al. 2005).

Studies on CCR1−/− mice documented the importance of the CCR1 in local infiltration of neutrophils and macrophages (Furuichi et al. 2008). Our data showing increased expression of CCR1 in NP are in accord with the recently described increased expression of the CCR1 ligand, C-C chemokine CCL23 in NP and chronic rhinosinusitis (Poposki et al. 2011). It has been shown that CCL23 in NP is produced by the eosinophilic infiltrate (Poposki et al. 2011), thus representing a link between the CCR3 (eosinophils) and CCR1 expressions in NP. In addition, the C-C chemokine CCL23 was reported not only to promote chemotaxis via CCR1 but also angiogenesis and endothelial cell migration (Hwang et al. 2005). These data thus correspond with our observation of low-level endothelial expression of CCR1.

Several of the cytokines that are related to accumulation of CCR1- and CCR3-positive cells are produced by epithelial cells, e.g., RANTES, eotaxin, IL-1β, IL-8, TNF-α, TGF-β, and GM-CSF (Stadnyk 2002; Xaubet et al. 1994). Mucosal surfaces and epithelial cells represent both a barrier and interface for the outer environmental stimuli (Tlaskalová-Hogenová et al. 1998). Epithelial cells express many key innate surface receptors such as TLRs and CD14 (Lin et al. 2007; Wang et al. 2007a, b; Funda et al. 2001) and are thus capable of recognition of various "danger" stimuli from the outer environment leading to activation of NFκ-B and induction of proinflammatory cytokines as well as

chemokines such as IL-8. We have reported increased expression of iNOS and insulin-like growth factor-1 receptor in NP (Fundová et al. 2008). Our hypothesis is that local dysregulation of homeostasis of epithelial cells in response to various environmental stimuli in genetically predisposed individuals may represent an important pathogenic mechanism in NP.

Apart from documenting an increased number of CCR3-positive cells in the stroma, we also found increased expression of CCR3 within the epithelial layer of NP (Table 1, Fig. 1). It seems that several chemokines and cytokines as well as mechanisms may contribute to the increased expression of CCR3 in NP. GM-CSF produced by epithelial cells (Shin et al. 2003; Roca-Ferrer et al. 1997) and IL-5 produced by activated T cells and eosinophils (Bachert et al. 1997) promote eosinophil activation and survival. Although GM-CSF alone does not directly affect the adhesion and degranulation of eosinophils, it can enhance eosinophil activation in the presence of cofactors such as VCAM-1 and ICAM-1 (Shin et al. 2003). Interesting observation about an autocrine production of IL-5 (Bachert et al. 1997; Simon et al. 1997) and RANTES by eosinophils may explain another contributing mechanism towards chronic accumulation of CCR3^{+} eosinophils in the inflammatory infiltrate of NP (Bachert et al. 2002). Eosinophils have been also reported as classical antigen-presenting cells (Wang et al. 2007a, b), an important feature that has not yet been addressed in relation to pathogenesis of NP.

In conclusion, locally dysregulated production of chemokines and cytokines may participate in pathogenesis of NP

by facilitating increased migration, accumulation, as well as activation of the immune cells expressing corresponding receptors. In this study, we document an increased expression of chemokine receptors CCR1 and CCR3 within cellular infiltrate as well as increased expression of CCR3 within the epithelium of nasal polyp biopsies that may contribute to the chronic status and inflammatory pathogenesis of NP. A blockage of effective expression of CCR1 or CCR3, or alternatively, a local depletion of infiltrating CCR1- or CCR3-positive cells may be a promising therapeutical approach in controlling this chronic inflammatory process in nasal mucosa.

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3.2. Publication II.

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Several studies in pathogenesis of NP and CRS focused on the inflammatory infiltrate, proinflammatory cytokines and chemokines and/or adaptive immune responses (Pawankar and Nonaka, 2007; Bachert et al., 2003). Less attention has been given to the nasal epithelium, an important part of the mucosal barrier and a contact surface with the outer environment, as well as innate immune mechanisms.

In this study we examined expression of insulin-like growth factor-1 receptor (IGF-1R) in nasal polyps and showed statistically significantly increased numbers of IGF-1R-positive cells in both stroma and epithelium of NP compared to nasal mucosa. Multiple factors such as EGF, KGF, TGF-β1 and also IGF-1 control homeostasis of epithelial cells (Dignass, 2001; Little et al., 2007). We think the increased expression of the receptor for insulin-like growth factor-1 in NP may be connected with prolonged survival or increased proliferation of epithelial cells that both have been described in NP (Coste et al., 1996; Mendelsohn et al., 2001). However, many more studies on the role of epithelium integrity and repair mechanisms are needed to understand inflammatory processes of the nose (Yan et al., 2013).

Epithelial cells were shown to display various immune function such as expression of MHC II molecules (Blend 1998), production of multiple cytokines and chemokines (Stadnyk, 1994; Striz et al., 2014) as well as expression of their receptors (Krasna et al., 2005). Epithelial cells also express innate immune receptors such as various TLRs and CD14 (Cario and Podolsky, 2000; Funda et al., 2001). One of the innate immune mechanisms that control various infections entities is production of nitric oxide by cytokine inducible nitric-oxide synthase (Nathan and Shiloh, 2000). Thus, we investigated the expression of inducible nitric-oxide synthase (iNOS) in biopsies of nasal polyps and nasal mucosa and found substantially increased number of iNOS-positive cells in both stroma and within epithelium of NP.

We think our results support recent opinion that both epithelial cells as well as innate immune mechanisms - as the first line of defense against pathogens and

environmental agents, may play important role in pathogenesis of NP (Kato, 2015; Hamilos, 2014).

Expression of IGF-1R and iNOS in Nasal Polyps; Epithelial Cell Homeostasis and Innate Immune Mechanisms in Pathogenesis of Nasal Polyposis

P. Fundová[a], T. Filipovský[a], D.P. Funda[b], O. Hovorka[b], R. Holý[a],
M. Navara[a], H. Tlaskalová-Hogenová[b]

[a]*Ear, Nose and Throat (Otorhinolaryngology) Department, Central Military Hospital, 169 02 Prague, Czech Republic*
fax   +420 973 202 734
e-mail   petra.fundova@uvn.cz

[b]*Division of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic*

ABSTRACT.   Nasal polyps (NP), edematous projections of nasal mucosa (NM), are characterized by an inflammatory cellular infiltrate, however, little is known about etiopathogenesis of NP. Both innate immune mechanisms leading to activation of NF-κB and homeostasis of epithelial cells were implicated in the pathogenesis of NP. In this study we investigated the expression of insulin-like growth factor-1 receptor (IGF-1R) and inducible nitric-oxide synthase (iNOS) in NP compared to healthy NM in both the epithelial and stromal compartments. Using immunohistochemistry, frozen tissue sections of NP from 18 patients, and mucosal biopsy specimens of the inferior turbinate from 17 subjects were stained for IGF-1R and iNOS markers. Fluorescence microscopy and computerized image analysis revealed low numbers of IGF-1R-positive cells in all specimens. However, substantially increased numbers of IGF-1R-positive cells were found in NP compared to NM both within the epithelium (1.63 *vs*. 0.43) and stroma (3.27 *vs*. 1.03). Positivity for iNOS was detected within the epithelium of NP compared with NM. Numbers of iNOS-positive single cells were highly increased in NP *vs*. NM in both epithelial (3.83 *vs*. 1.08) and stromal (4.96 *vs*. 2.67) compartments. An increased iNOS expression within the epithelial layer as well as increased number of iNOS- and IGF-1R-positive cells in NP was observed. This suggests that innate immune mechanism, and to a lesser extent also growth and homeostasis of epithelial cells, may play a role in formation of NP.

Abbreviations

CD	cluster of differentiation	NF-κB	nuclear factor κB	NP	nasal polyps
H&E	hematoxylin & eosin	NM	nasal mucosa	PBS	phosphate-buffered saline
IGF-1R	insulin-like growth factor-1 receptor	NOS	nitric-oxide synthase	TBS	Tris-buffered saline
IL	interleukin	iNOS	inducible NOS		

Nasal polyposis is a recurrent affletion of NM characterized by chronic inflammatory processes but its etiology and pathogenesis remain unclear (Bachert *et al.* 2003). Nasal polyps consist of loose connective tissue, edema, some glands and capillaries and inflammatory cells infiltration comprising eosinophils, neutrophils, mast cells as well as lymphocytes with increased proportion of T cells (Jones *et al.* 1987; Fokkens *et al.* 2007). In European population the prevalence of the NP ranges from 1.4 to 4.3% (Fokkens *et al.* 2007).

Nasal epithelium plays an important role in the mucosal barrier function, protecting NM from various environmental agents. Epithelial cells display various immune functions as they can produce many different cytokines (Stadnyk *et al.* 1994), express MHC II molecules and may act as antigen-presenting cells (Bland 1988; Bleicher *et al.* 1990). Epithelial cells take also an important part in mucosal innate immune mechanisms as they can express various innate immune receptors, such as CD14 and Toll-like receptors, and represent a potential source of soluble sCD14 (Cario *et al.* 2000; Funda *et al.* 2001). Multiple factors control epithelial cell homeostasis including involvement of IEL lymphocytes (Boismenu *et al.* 1994) together with various growth factors, such as keratinocyte growth factor (KGF), epidermal growth factor (EGF), transforming growth factor (TGF-β_1), and insulin-like growth factors (IGFs) (Dignass *et al.* 2001). While the effect of TGF-β_1 in nasal polyposis was studied by Little *et al.* 2007, the role of IGF-1 and especially its receptor (IGF-1R) has not yet been addressed.

Various toxic and infectious stimuli encountered at the level of NM may activate innate immune mechanisms and lead to induction of pro-inflammatory cytokines. The production of nitric oxide from L-argi-

nine by the cytokine-inducible (or type 2) iNOS is one of the key defense mechanisms of mammalian phago-cytes (Nathan *et al.* 2000). Common mucosal system is constantly exposed to various environmental agents, *e.g.*, to allergens, and to viral and bacterial agents. In rodents, the control of many different infectious patho-gens (including viruses, bacteria, protozoa, and fungi) is dependent on the expression of iNOS activity with iNOS-induced nitric oxide exerting a direct antimicrobial effect (Fang *et al.* 1997).

We hypothesize that a defect in homeostasis of epithelial cells and/or innate immune mechanisms and subsequent NF-κB activation followed by induction of pro-inflammatory factors may play an important part in pathogenesis of nasal polyposis. Thus, we examined expression of IGF-1R and iNOS in NP compa-red to healthy NM. Using immunohistochemistry and quantitative microscopic analyses we assessed both the pattern of expression as well as quantified number of positive cells.

MATERIALS AND METHODS

Subjects and specimens. We compared 18 NP biopsies obtained from 18 patients with bilateral NP and 14 NM biopsies from 14 of those patients. In addition, 3 NM biopsies from healthy volunteers were also investigated. None of the patients received glucocorticoid treatment during the at least 1-year period prior to the biopsy. Informed consent for taking the biopsies was obtained from all the patients. The study was acknowledged by the *Ethical Committee of the Central Military Hospital*, Prague.

Biopsies of ≈4 × 3 × 2 mm were harvested from the antero–inferior part of the inferior turbinate and from the most anterior polyp using 10 % xylocaine aerosol as local anesthetic. The biopsies were imme-diately embedded in OCT compound (Tissue-Tek; *Miles Scientific*, USA), snap frozen in 2-methylbutane (*BHD Laboratory Supplies*, UK) and stored at –80 °C. Cryosections were cut serially at 4-mm, dried over-night at room temperature, fixed in acetone for 10 min and used for immunostaining.

Immunohistochemical staining. The primary incubation with mouse anti-human monoclonal anti-bodies (Abs) was performed at room temperature for 1 h in the dark, then the slides were rinsed 3× for 5 min in TBS. The following primary mAbs were used: purified anti-IGF-IRa (*Pharmingen*, USA), biotin-conjugated anti-iNOS (*Tranduction Labs*, USA). The secondary incubation was performed at room tempe-rature in the dark with biotin-conjugated goat anti-mouse IgG F(ab´)$_2$ mAb (*Jackson IR Lab.*, USA). The sections were then washed 3× for 5 min in TBS and incubated for 1 h in the dark with streptavidin–CY3 or –FITC dilution 1 : 100 (*Jackson IR Lab.*). Sections were counterstained with hematoxylin and mounted in aqueous mounting medium (Faramount; *DAKO*, Denmark). Incubation without primary antibodies served as negative control.

Cell counting and computer image analysis. All tissue sections were coded and blindly examined in both epithelial and lamina propria compartments. Positive cells were counted under the *Olympus* fluores-cence microscope (AX70 Provis; *Olympus*, Japan) using a calibrated eyepiece graticule as well as by com-puter image analysis (analySIS® 3.0; *Soft Imaging System*, Germany) using a MEGAII digital camera. The magnification was matching that used with the calibrated eyepiece graticule. The number of positive cells was counted in 20 randomly selected fields and in 20 pictures corresponding to the calibrated eyepiece graticule fields in both lamina propria and epithelial compartments.

Statistical analysis. Data are given as mean ±SEM, and the level of significance was assayed by unpaired *t*-test and analysis of variance (ANOVA) followed by the Bonferroni test.


RESULTS

Expressions of IGF-1R and iNOS were evaluated in a total of 18 NP biopsies and 17 NM biopsies (Table I). In both IGF-1R and iNOS staining, no differences in the number of positive cells as well as patterns of expression were detected between biopsies of healthy NM from the inferior turbinate taken from patients with NP (*n* = 14) and NM from healthy volunteers (*n* = 3). Data for NM (*n* = 17) comprise healthy NM from both groups.

Biopsy specimens were examined under fluorescence microscope and quantitative analysis of posi-tive cells was performed by using calibrated eyepiece graticule as well computer image analysis. Only scat-tered and low numbers of IGF-1R positive cells were found in all specimens and immunohistochemical staining revealed no changes in the pattern of expression in IGF-1R in NM compared to NP. However, we found consistently increased numbers of solitary IGF-1R positive cells within both the lamina propria (3.27 ± 0.08 *vs.* 1.03 ± 0.05, $p < 10^{-4}$) as well as the epithelial (1.63 ± 0.06 *vs.* 0.43 ± 0.03, $p < 10^{-4}$) compartments

Table I. Number (means ± SEM[a]) of IGF-1R-positive and iNOS-positive cells[b] in NP *vs.* NM

Specimen		Nasal polyps	Nasal mucosa
IGF-1R–NP *vs.* NM	epithelium	1.63 ± 0.06	0.43 ± 0.03
	stroma	3.27 ± 0.08	1.03 ± 0.05
iNOS–NP *vs.* NM	epithelium	3.83 ± 0.13	1.08 ± 0.07
	stroma	4.96 ± 0.13	2.67 ± 0.09

[a]$p < 10^{-3}$; from NM within the same tissue compartment.
[b]Calculated as described in *Materials and Methods*.
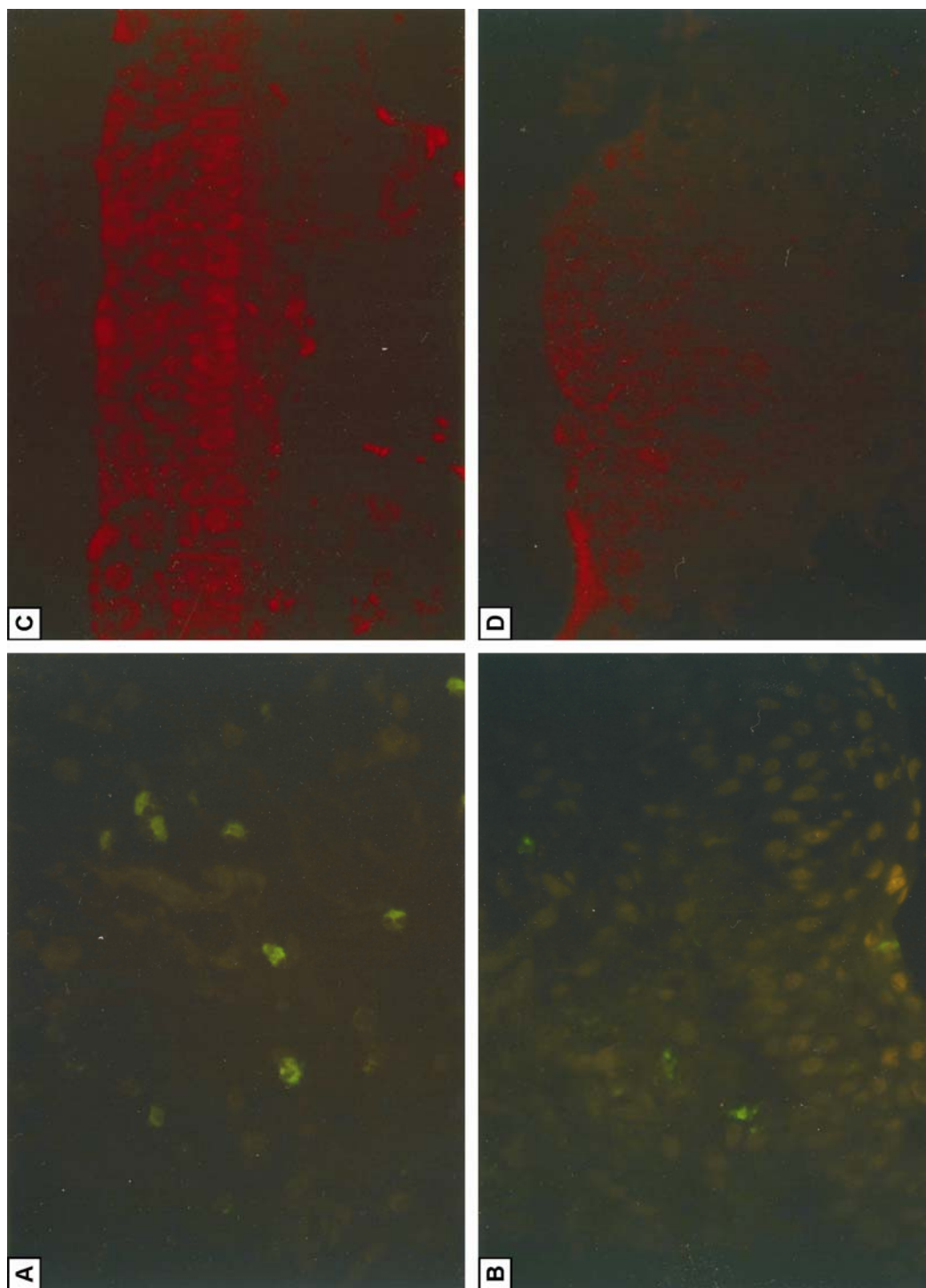
Fig. 1. Immunofluorescence detection of IGF-1R-positive cells in NP (**A**) compared to healthy NM (**B**); **C**, **D**: immunofluorescence positivity for iNOS within a biopsy of NP and NM, respectively (magnification all ×256).

of NP *vs*. NM (Table I, Fig. 1). The IGF-R1 positivity was detected on single, isolated cells within the epithelium and lamina propria compartments.

Fluorescence microscopy revealed iNOS positivity in epithelial cells of NP compared to NM (Fig. 1). Single stained cells were detected within the epithelial compartment and these were counted in the quantitative assessment of the iNOS expression (Fig. 1B). Thus, apart from increased iNOS positivity of epithelial cells in NP, substantially increased numbers of iNOS-positive single cells were found in NP *vs*. NM in both epithelial (3.83 ± 0.13 *vs*. 1.08 ± 0.07, $p < 10^{-4}$) and stromal (4.96 ± 0.13 *vs*. 2.67 ± 0.09, $p < 10^{-4}$) compartments (Fig. 1B–D).

DISCUSSION

We established a significantly increased number of IGF-1R and iNOS positive cells in both lamina propria and epithelial compartments of NP compared to physiological NM (Table I). Increased epithelial positivity for iNOS was detected in NP biopsies (Fig. 1). This is in accordance with several papers documenting immuno-inflammatory infiltrate in NP. The massive influx of eosinophils as well as other leukocytes is facilitated by increased endothelial expression of ICAM-1 in NP (Tingsgaard *et al*. 1998). Furthermore, expression of ICAM-1 is regulated by NF-κB (Mannin *et al*. 1995). The cellular infiltrate of NP comprises of T cells, antigen-presenting cells and to a much lesser extend also B cells (Ihan *et al*. 1997). Within the inflammatory infiltrate increased number of CD45RO$^+$ memory T cells as well as HLA-DR$^+$ cells (including expression on epithelium) were documented in NP (Linder *et al*. 1993). P- and to lesser extend also L-selectins were shown to mediate adhesion and homing of peripheral blood lymphocytes in NP (Symon *et al*. 1999). Increased levels of cytokines IL-6, IL-8, and the chemokine RANTES were reported in both atopic and non-atopic NP (Teran *et al*. 1997; Allen *et al*. 1997). However, the triggering events that lead to this chronic inflammatory disease of NM are not known.

Our hypothesis is that one possible mechanism in the formation of NP is a defect in the homeostasis of epithelial cells in response to various environmental stimuli. These could include various toxic or infectious environmental entities and/or chronic changes in commensal microflora at the level of nasal mucosa (Tlaskalová-Hogenová *et al*. 2004). IGF-1R is promoting prolonged cell survival and was also implicated in tumorigenesis (Mendelsohn *et al*. 2001). Furthermore, increased proliferation of epithelial cells has been well documented in NP (Coste *et al*. 1996). The increased expression of IGF-1R, on single epithelial cells and as well as within the cellular infiltrate of NP, reported here, represents a molecular basis for a hypertrophy of NM in NP. It remains to be determined, to which extent a disruption of epithelial cell homeostasis is a primary factor predisposing to the nasal polyposis or an outcome of various chemokines, cytokines and growth factors produced by the infiltrating inflammatory cells.

Epithelial cells on mucosal surfaces are in the first line of defense and/or recognition of various environmental stimuli that may lead to activation of innate immune mechanisms and NF-κB-dependent induction of pro-inflammatory cytokines, chemokines and iNOS. We found increased positivity for iNOS in the epithelium of NP compared to NM, which confirms the findings of Watkins *et al*. (1998), who have documented increased epithelial iNOS mRNA expression in NP and verified these findings on protein level by immunohistochemistry on paraffin-embedded tissue blocks. On the other hand (and in contrast with the above-mentioned paper), we have also found high iNOS expression (Table I, Fig. 1) on the subset of scattered cells within stroma and to a lesser extent within epithelium of NP, probably infiltrating inflammatory cells. This is a new finding as regards iNOS expression in NP. The difference between these two immuno-histochemical detections is probably due to the fact that the original study was done on paraffin-embedded tissue with antigen retrieval, as well as due to a limited number of NP specimens (Watkins *et al*. 1998). Our result together with the data of Kang *et al*. (2004) are in striking contrast with the rather surprising data of Ramis *et al*. (2000), who reported no difference in iNOS expression as well as NF-κB activity in NP compared to healthy NM.

Our data document and confirm an increased iNOS expression within the epithelial layer of NP and newly report an increased number of iNOS and IGF-1R-positive cells in NP. This suggests that innate immune mechanism, *e.g*. signaling through Toll-like receptors and induction of NF-κB, regulate inflammatory factors including iNOS and, to a lesser extent, also a defect in homeostasis of epithelial cells, *i.e*. prolonged cell survival, may play a role in pathogenesis and formation of NP.

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This review covers the topic of the first, more clinically focused part of my thesis and is intended as an overview on history and pathogenesis of nasal polyposis for Czech ENT (and other) clinicians dealing with NP in their routine outpatient clinics. It consists of chapters covering basic characteristics and history of the disease, that has been already noted and reported in the ancient Egypt almost 4500 years ago (Pahor and Farid, 2003), but until now its pathogenesis and causative treatment remain unsolved. Next chapters covers the data on prevalence of NP, also in relation to asthma, the contribution of genetic factors and possible environmental factors that were considered but not confirmed in etiology of NP. The next three paragraphs then deal with possible immune mechanisms involved in pathogenesis of NP - role of mucosal immunity and epithelial cells, innate immune mechanisms, and chemokines and cytokines. One recent topic not covered by this review is the relation of nasal microbiom to development of NP and CRS. While role of microbiota is studied in many other diseases for relatively long period, only very few attempts have been made to study the nose microbiom in relation to local pathogenic processes (Choi et al., 2014; Chalermwatanachai et al., 2015).

Patogenetické mechanismy u nosní polypózy

Pathogenetic mechanisms in nasal polyposis

PETRA FUNDOVÁ[1], MICHAL NAVARA[1], HELENA TLASKALOVÁ-HOGENOVÁ[2]

[1]*Ústřední vojenská nemocnice, Praha*
[2]*Oddělení imunologie a gnotobiologie, MBÚ, AV, Praha*

SOUHRN

Nosní polypy (NP) jsou častým a historicky velmi dlouho známým onemocněním nosní sliznice, jehož etiologie je neznámá a pouze v poslední době jsou komplexněji identifikovány možné patogenetické mechanismy. Tento přehledný článek zmiňuje faktory prostředí a genetické predispozice u NP a zaměřuje se na shrnutí dosavadních důležitých poznatků ve vztahu k patogenezi NP (tohoto onemocnění), především pak na úlohu slizniční imunity, mechanismy přirozené imunity, homeoastázu slizničního epitelu a roli růstových faktorů, cytokinů a chemokinů v rozvoji tohoto chronického, recidivujícího, zánětlivého onemocnění nosní mukózy.
Klíčová slova:

SUMMARY

Nasal polyps (NP) are a common and historically long known disease of nasal mucosa with unknown etiology. Recently, the complexity of possible pathogenetic mechanisms started to be identified. This article discusses environmental factors and genetical predisposition in NP and reviews the current knowledge in relation to pathogenesis of NP. It focuses particularly on the role of mucosal immune system, the innate immune mechanisms, epithelial homeostasis and the role of growth factors, cytokines and chemokines in the development of this chronic recurrent inflammatory disease of the nasal mucosa.
Key words: nasal polyps, pathogenesis, mucosal imunity, epithelial cells, innate imunity, eosinophils, cytokines, chemokines, inflammation

Úvod

Nosní polypóza je chronické a recidivující zánětlivé onemocnění sliznice nosu a vedlejších nosních dutin, projevující se nenádorovými edematózními zduřeními zanícené sliznice prolabujícími do nosu. Může se jednat o jeden solitární polyp až po mnohočetné polypy vyplňující vedlejší dutiny nosní, i celou nosní dutinu, v extrémních případech i prolabující vpředu z nosních průduchů. Toto vede k nosní obstrukci, nosní sekreci, bolestem hlavy, dysosmii, případně i anosmii a celkově k snížené kvalitě života (Wabnitz et al., 2005). Nosní polypy jsou charakterizovány volnou pojivovou tkání, edémem, sníženým množstvím žlázek a cév, žádnými nervovými strukturami a dlaždicovou metaplazií povrchového epitelu (Taylor, 1963). Stroma zralých nosních polypů sestává kromě edému z podpůrných fibrobastů a infiltrujích buněk, z nichž asi 80 % představují aktivavané eosinofily (Stoop, 1993), dále neutrofily, žírné buňky a lymfocyty se zvýšeným podílem T-lymfocytů (Jones et al., 1987; Fokkens et al., 2007). Eosinofily jsou lokalizovány podél cév, žláz a pod bazální membránou epitelu (Kakoi, 1987).

Historie

První zmínka o nosních polypech je doložena v lékařských spisech ze starého Egypta kolem r. 2475 př. n. l.

(Pahor et al., 2003), další následují z byzantského období (324–1453 n. l.). Originální řecky psané texty ze čtvrtého století podrobně rozebírají definici, příznaky a konzervativní léčbu zahrnující lokální aplikaci látek v olejovém základu či instilaci kaustických látek (Lascaratos et al., 2000).

Prevalence

Prevalence nosních polypů se pohybuje v rozmezí 0,5–4,5 % a liší se v jednotlivých geografických oblastech a velmi pravděpodobně, též v závislosti na genetické predispozici u různých ras a populací. Výskyt onemocnění stoupá s věkem a je častější u mužů (2,2:1) (Johansson et al., 2003), s výjimkou Samterovy triády, kde jsou častěji postiženy ženy (2,3:1), (Szczeklik et al., 2000). Podle celonárodního průzkumu v Koreji byla prevalence nosních polypů, diagnostikovaných přední nosní endoskopií 0,5% v celkové populaci (Min et al., 1996). Švédská populační studie diagnostikující nosní polypy pomocí přední endoskopie, udává prevalenci onemocnění 2,7 % v celkové populaci, s vyšším výskytem u mužů a u astmatiků (Johansson et al., 2003). Finská studie založená na dotazníku udává prevalenci 4,3 % u dospělé populace (Hedman et al., 1999). Retrospektivní studie hodnotící data 6000 pacientů z nemocnic, alergologických klinik a ambulancí v USA udávala prevalenci 4,2% v populaci, a 6,7% u astmatiků (Settipane et al., 1977). Výskyt polypů u dětí je výjimečný, s výjimkou asociací s cystickou fibrózou (Crockett et al., 1987).

Genetické faktory a asociace s jinými onemocněními

Familiární zátěž u NP je častá. Ve francouzské multicentrické studii udávalo 58,7% pacientů, že mají jednoho, nebo více přímých příbuzných (rodiče, děti, sourozenci) postižených NP, 43,6% pacientů mělo jednoho, nebo více přímých příbuzných trpících astmatem (Crampette et al., 2001). Podobně jako u autoimunitních onemocnění byla u NP popsána asociace s určitými HLA alelami. Například maďarská studie srovnávající 50 pacientů s NP s 50 zdravými jedinci ukazovala 2–3× vyšší pravděpodobnost vývinu nosní polypózy u jedinců nesoucích HLA-DR7-DQA1*0201 a –DQB1*0202 (Molnar-Gabor et al., 2000), zatímco mexická studie na vzorku 31 pacientů s NP vůči 151 zdravým jedincům udávala 5,53krát vyšší riziko vývoje nemoci u jedinců nesoucích alelu HLA-DR7-DQA1*0201 a –DQB1*0201 (Fajardo-Dolci et al., 2006). Podle čínské studie srovnávající HLA alely u 31 pacientů s NP vůči 81 zdravým jedincům představující HLA-DR16, HLA-DQ8, a HLA-DQ9 pro své nositele zvýšené riziko onemocnění, kdežto determinanta HLA-DQ7 může naopak být spojena s vyšší odolností vůči nemoci (Zhai et al., 2007). Kromě HLA asociací a zvýšené rodinné zátěže se však nepodařilo identifikovat jiné výrazné genetické faktory a např. genová terapie se tak jeví jako velmi nepravděpodobná budoucí kauzální terapie nosních polypů. Celkově lze uzavřít, že faktory prostředí mají rozhodující vliv na rozvoj onemocnění u geneticky predisponovaných jedinců.

Z populačních studií vyplývá, že NP se vyskytuje s vyšší prevalencí u pacientů s bronchiálním astmatem (Hedman et al., 1999; Settipane et al., 1977). Ve studiích, zahrnujících velké skupiny pacientů s NP, bylo astma nalezeno ve 30-60% (Slavin, 1982; Larsen, 1996). Nespecifická bronchiální hyperreaktivita (BHR) a/nebo astma se vyskytují častěji u pacientů s nosními polypy než v ostatní populaci (Slavin, 1982). Alergii jako možnou příčinu nosních polypů uvedl Younge ve své původní práci publikované v BMJ v roce 1907 (Younge, 1907). Od té doby byla opakovaně citována, nicméně pozdější publikované práce tuto značně rozšířenou teorii nepotvrzují (Mygind, 2000).

Aspirinová intolerance, asociovaná s bronchiálním astmatem a nosními polypy, je rozeznávána jako klinická jednotka, označovaná aspirinová, neboli Samterova, nebo také Widalova triáda (Samter M et al., 1967, 1968), a vyskytuje se s prevalencí 10–20 % v astmatické populaci (Hedman et al., 1999; Samter et al., 1968, Kasper et al., 2003). U žen, které převažovaly muže v poměru 2,3:1, se výskyt symptomů objevil signifikantně dříve a nemoc byla progresivnější a závažnější než u mužů (Szczeklik et al., 2000). Cystická fibróza je nejčastější letální autosomálně recesivní onemocnění běloše populace (Henriksson, 2002). V ORL oblasti se manifestuje chronickou sinusitidou a nosními polypy. Prevalence nosních polypů u pacientů s cystickou fibrózou se odhaduje mezi 7–56 % s vyšší prevalencí při použití nosní endoskopie při vyšetření (Cepero et al., 1987; Taylor et al., 1974; Brihaye et al., 1994; Jones et al., 1993). Pro výše uvedené případy jsou typické rekurentní nosní polypy, vyžadující dlouhotrvající, nebo i trvalou kortikoidní terapii (Cepero et al., 1987; Szczeklik et al., 2000). Ačkoliv se nosní polypy často sdružují s jinými onemocněními, je nepravděpodobné, že jsou kauzálním projevem těchto onemocnění.

Faktory vnějšího prostředí

Jako možné vyvolávající agens nosních polypů byly uváděny faktory prostředí jako alergeny potravinového charakteru, polutanty, inhalanty a chronická infekce virová, bakteriální či mykotická. U hlodavců bylo prokázáno, že kontrola mnoha různých infekčních patogenů (včetně virů, bakterií, protozoárních infekcí a mykóz) je v přímé závislosti na expresi iNOS s iNOS indukovaným NO, majícím přímý antimikrobiální efekt (Fang, 1997). Zvýšená exprese iNOS pozitivních buněk v nosních polypech ve srovnání s nosní sliznicí tedy poukazuje na vliv vnějšího prostředí (Fundová, 2008). Role infekčních podnětů je považována za významnou, zejména kmeny *Streptococcus pneumoniae, Staphylococcus aureus* nebo *Bacteroides fragilis* (obvyklých patogenů u sinusitidy) nebo *Pseudomonas aeruginosa*, často nacházeném při cystické fibroze (Norlander, 1993). V současnosti je velmi diskutován podíl superantigenů, odvozených zejména ze *Staphylococcus aureus*, na sekreci IgE B buňkami, stejně jako přímý vliv superantigenů na charakteristické buňky

zánětlivého infiltrátu NP, jako jsou eosinofily (Bachert, 2008). Nicméně, doposud nebylo identifikováno žádné jednotlivé (ani skupina příbuzných) agens u NP a je otázka, zda vůbec specifické etiologické agens existuje, či zda různé noxy vnějšího prostředí mohou vést k rozvoji NP u geneticky predisponovaných jedinců.

Role slizničního imunitního systému (a epitelových buněk)

Slizniční imunitní systém hraje zásadní roli v interakci organismu s vnějším prostředím. Sliznice je hlavním indukčním místem pro navození tolerance k rozpustným antigenům (Weiner, 1997). Interakce mezi adhezivními molekulami podskupin β2- a β7 integrinů a jejich ligandů pravděpodobně hraje důležitou roli v slizniční imunitní odpovědi horního dýchacího traktu (Demoly, 1998, McNulty, 1999). Slizniční bariéra nebo imunitní exkluze je udržována produkcí velkého množství sekrečního IgA (sIgA), (Mestecky, 1987). Komenzální mikroflóra hraje důležitou roli v indukci produkce sekrečního IgA a jeho specifit (Fagarasan, 2006). Případné změny spektra specifit sIgA u onemocnění, která postihují sliznice, nejsou doposud dobře známy. Komenzální mikroflóra, která kolonizuje sliznice, je v symbiotické koexistenci s makroorganismem a patří mezi komplexní přirozené mechanismy zajišťující imunitu organismu proti patogenním mikroorganismům. Bakteriální populace tvoří komplexní ekosystémy. Pokud je jejich složení optimální, mikroflóra zabraňuje adhezi a množení patogenních mikroorganismů (Tlaskalová, 2002, 2004). V jiných kompartmentech slizničního imunitního systému má komenzální mikroflora významnou úlohu v indukci slizničních humoralních i buněčných imunitních odpovědí a je též významným faktorem ovlivňujícím vývoj regulačních slizničních imunitních mechanismů, včetně neopovídavosti „tolerance" imunitního systému na cizorodé antigeny prostředí, které nezpůsobují ohrožení organismu (Tlaskalová, 2004; Cebra, 1999). Na rozdíl od střevního traktu, komenzální mikroflóra nosní sliznice nebyla doposud adekvátně studována.

Důležitým faktorem v patogenezi NP by mohla být porucha homeostázy epitelových buněk. Epitelové buňky, které jsou na nosní sliznici kontinuálně exponovány mnoha faktory vnějšího prostředí, tvoří důležitou součást slizničního imunitního systému neboť produkují široké spektrum cytokinů a chemokinů (Stadnyk, 1994), exprimují MHC glykoproteiny II. třídy (basolaterální exprese) stejně jako i neklasické MHC glykoproteiny I. třídy (CD1d, TLA), a mohou sloužit jako antigen-prezentující buňky pro intraepitelové lymfocyty (IEL) (Bleicher, 1990; Tlaskalova, 2004). IEL představují unikátní populaci lymfoidních buněk, neboť se vyvíjejí mimo thymus a často exprimují tkáňově specifický gama/delta T-buněčný receptor a CD8αα homodimer. IEL hrají významnou roli v udržování periferní tolerance (Locke, 2006) a také v regulaci růstu epiteliálních buněk, a to produkcí růstového faktoru keratinocytů (keratinocyte growth factor [KGF]), který společně s dalšími růstovými faktory (epi-

dermal growth factor [EGF], transforming growth factor β [TGF-β], a insulin-like growth factors [IGFs]) zajištuje integritu a homeostázu slizničního epitelu (Boismenu, 1996; Dignass, 2001). V naší studii jsme prokázali zvýšený počet buněk exprimujících receptor pro insulin-like growth factor 1 (IGF-1R) v nosních polypech ve srovnání s nepostiženou nosní sliznicí (Fundová, 2008). IGF-1 prodlužuje životní cyklus buňky a zvýšená exprese by mohla přispívat k hypertrofii sliznice u NP. U nosní polypózy byla též popsána zvýšená proliferace epitelových buněk (Coste, 1996).

Mechanismy přirozené imunity

První linie obrany proti patogenům a/nebo vnějším škodlivinám je zprostředkována rychlými neadaptivními imunitními odpověďmi. Epitelové buňky jsou kromě funkce mechanické bariéry a účasti mechanismech slizniční imunity (prezentace antigenu, transport sIgA) také důležitým prvkem přirozené imunity, protože mohou exprimovat receptory přirozené imunity jako např. koreceptor pro lipopolysaccharide CD14 a Toll-like receptory (TLR) (Cario a Funda, 2001) a podílet se tak na indukci mediátorů zánětu.

TLR jsou klíčové receptory přirozené imunity a reprezentují důležitou spojnici mezi přirozenou a specifickou imunitou (Akira, 2001). Rozeznávají různé cizorodé, zejména fylogeneticky konzervované složky mikroorganismů „microbe associated molecular patterns" (MAMP) a pravděpodobně také vlastní „nebezpečí signalizující" antigeny (Tlaskalová, 2004). Signalizace přes TLR vede k aktivaci nukleárního faktoru kappaB a následne indukci prozánětlivých cytokinů, chemokinů, indukovatelné NO syntetázy (iNOS – inducible nitric oxide synthase) a zvýšené expresi některých adhezivních a kostimulačních receptorů na povrchu antigen prezentujících buněk (APC) (Akira, 2001; Shibole, 2007). Oxid dusnatý (NO) je antimikrobiální faktor vznikající působením NO syntetáz a jeden z důležitých nástrojů rychlé obranné reakce organismu. Sledovali jsme expresi iNOS v biopsiích NP a zjistili jsme, že je ve srovnání s nepostiženou nosní sliznicí zvýšená (Fundová, 2008).

Na tkáňových kulturách epitelových nosních buněk izolovaných z nosních polypů, byla prokázána zvýšená exprese TLR3 (Wang, 2007). TLR signály v žírných buňkách zvyšují uvolňování Th2 cytokinů, predvsim pak IL-5 (Iwamura, 2008; Pawankar, 2005). Ačkoliv tyto odpovědi hrají důležitou ochrannou funkci proti infekci, mohou též stát na počátku dysregulace slizničních imunitních odpovědí na antigeny vnějšího prostředí u geneticky predisponovaných jedinců. Při srovnávání vzorků 15 nosních polypů a 15 vzorků nepostižené nosní sliznice jsme pozorovali zvýšenou expresi TLR3, TLR4 a TLR5 u NP a nenašli žádné rozdíly v expresi TLR1, 2, a TLR6 (Fundová et al., nepublikované výsledky). Změny exprese jednotlivých TLR u nosní polypózy ukazují na podíl faktoru prostředí a selektivní exprese pouze určitých TLR by mohla pomoci identifikovat možné/á etiologické/á agens.

Cytokiny a chemokiny

Pro nosní polypy je, kromě zvýšené infiltrace zánětlivými buňkami, typická také zvýšená produkce řady prozánětlivých cytokinů a chemokinů, které mohou regulací migrace, přežívání a aktivace buněk zánětlivého infiltrátu výrazně přispívat k rozvoji NP a udržování chronického zánětu nosní sliznice. Na infiltraci eosinofily se významně podílí GM-CSF produkovaný epiteliálními buňkami nosní sliznice a nosních polypů (Xaubet, 1994). Chemokiny jako RANTES a eotaxin, zvýšené v nosních polypech, jsou také výraznými chemoatraktanty eosinofilů (Nonaka, 1999; Allen, 1998). Ačkoli samotný GM-CSF nemůže přímo ovlivnit adhezi a degranulaci eosinofilů a produkci superoxidového radikálu, v přítomnosti kofaktorů (VCAM-1, ICAM-1) může GM-CSF aktivaci eosinofilů zvyšovat (Shin, 2003). Kupříkladu TNF-a a IL-4/IL-13 mohou zvyšovat expresi VCAM-1 (vascular cell adhesion molecule-1) a tím usnadňovat migraci eosinofilů do tkáně (Pawankar, 2003). Cytokin IL-5, produkovaný aktivovanými T-lymfocyty a eosiofily zásadně prodlužuje přežívání eosinofilů inhibicí apoptózy (Bachert, 1997; Simon, 1997). Navíc eotaxin může působit v nosních polypech lokálně a zvyšovat poškození tkáně (Pawankar, 2003). V nosních polypech je rovněž zvýšená exprese IL-8, který působí chemotakticky na neutrofily. Navíc, prozánětlivé cytokiny jako IL-1β a TNF-α, zvyšující expresi ICAM-1 v endotelových buňkách, mohou navodit migraci T-buněk a neutrofilů do nosních polypů. Bylo také prokázáno zapojení P- a L-selektinů ve zprostředkování vazby T-buněk na sliznici u nosní polypózy (Symon, 1999).

Závěr

Nosní polypy jsou velmi častým onemocněním nosní sliznice, nicméně etiologické faktory a rozhodující patogenetické mechanismy nejsou zatím identifikovány. Ačkoliv byla u NP popsána asociace s určitými HLA alelami, nejedná se o výrazně geneticky podmíněné onemocnění s jasným typem dědičnosti a faktory vnějšího prostředí hrají pravděpodobně rozhodující úlohu. K dispozici není žádný dobře etablovaný zvířecí model nosní polypózy, a tudíž je velmi obtížné určit, co je příčina a co následek v etiopatogenezi NP. Nedostatek takovýchto znalostí činí kauzální léčbu a sekundární prevenci tohoto onemocnění dosud nedostupnou.

Imunitní funkce epitelových buněk jako např. produkce chemokinů a cytokinů, prezentace antigenů, transport sIgA, a poruchy homeostázy epitelové bariéry (proliferace a reparace epitelu nosní sliznice) nebyly doposud podrobně studovány u NP. Role infekčních podnětů je považována za významnou. Zvýšená exprese iNOS a určitých TLR toto tvrzení podporuje. Superantigeny, odvozené zejména ze *Staphylococcus aureus*, mohou výrazně ovlivňovat produkci IgE, a eosinofily. Přítomnost aktivovaných eosinofilů v tkáni je základní charakteristikou NP a je výsledkem komplexního sledu událostí, které regulují jejich influx z oběhu, stejně jako jejich pohyb, aktivaci a přežívání v tkáni. Chemokiny a cytokiny, produkované především epitelovými buňkami

a imunitními buňkami zánětlivého infiltrátu NP, se pravděpodobně výrazně podílejí v patogenezi, nicméně není jasné, nakolik jsou příčinou vzniku NP, a pokud ano, které signalizační kaskády hraji rozhodující úlohu.

Poruchy homeostázy a reparačních procesů epitelu nosní sliznice, podíl mechanismů přirozené imunity v reakci na chronickou stimulaci různými agens vnějšího prostředí, a dysregulace cytokinových a chemokinových signalizačních sítí představují v současnosti hlavní oblasti výzkumu v patogenezi NP.

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as. MUDr. Petra Fundová
Ústřední vojenská nemocnice
U vojenské nemocnice 1200
169 02 Praha 6
e-mail: petra.fundova@uvn.cz

3.1. Experimentally focused studies: mucosal immunity and environmental factors in autoimmune type 1 diabetes mellitus.

3.2.1. Publication IV.

Antvorskov JC, **Fundová P,** Buschard K, Funda DP. Impact of dietary gluten on regulatory T cells and Th17 cells in BALB/c mice. ***PLoS One***, **2012;7(3):e33315.** IF (2015) = 3.057

Environmental factors play a major role in the relatively recent increase of type 1 diabetes (T1D) in developed countries (Bach, 1994; Graves and Eisenbarth, 1999). The remarkable geographical difference (Diabetes Epidemiology Group, 1988) in the prevalence of the disease and the fact that more than 50% of identical twins are discordant for T1D also point to the importance of environmental factors. Furthermore, during a relatively short period of a few decades the incidence substantially increased, especially in the developed countries (Laakso et al., 1991).

Several studies in both NOD mice as well as BB rats have documented that diets influence diabetes incidence (Scot, 1996; Coleman et al., 1990). It is well documented that the diabetes-promoting agents are not carbohydrates but come from the plant protein fraction of natural diets (Hoorfar et al., 1991; Hoorfar et al., 1993). It has been shown that gluten-free diet highly decreases diabetes incidence in NOD mice (Funda et al., 1999; Schmid et al., 2004). Gliadin, the component of wheat gluten that triggers celiac diseases in susceptible individuals, was shown to activate innate immune mechanisms and also increase intestinal permeability (Drago et al., 2006; Jelinkova et al., 2004). Thus, gliadin has been also considered in pathogenesis of type 1 diabetes (Visser et al., 2009, Funda et al., 2008).

In this study we compared the effect of standard, gluten-containing diet and the diabetes preventive, gluten-free diet on various regulatory T cell subsets and Th17 cells in immunocompetent BALB/c mice. The diet was fed to the experimental animals prenatally and cell subsets were assessed by flowcytometry in mucosal (MLN, PLN, PP) compared to non-mucosal (spleen, ILN) lymphoid organs. The standard, gluten-containing diet led to general decrease of $\gamma\delta$ T cells in all lymphoid compartments studied. In type 1 diabetes, mucosal $\gamma\delta$ T cells were interestingly shown as regulatory or critical in oral tolerance induction (Harrison et al., 1996; Locke et al., 2006). The standard, gluten-containing diet also influenced proportion of $CD4^+CD45RBlow^+$ and $CD4^+CD45RBhigh^+$ T cells. Fewer

CD4$^+$ T cells expressed CD62L in PP and increased proportion of CD103$^+$ mucosa-homing T cells was noted in spleen and PLN. There was no effect dietary effect on CD4$^+$Foxp3$^+$ Tregs. Finally, significantly increased proportion of Th17 cells was detected in PLN of mice fed the standard diet.

In conclusion, we showed that the diabetes-permissive, standard diet versus gluten-free, diabetes-protective diets influence various T cell subsets e.g. γδ T cells and Th17 cells, preferentially in mucosal compartments.

PLoS one

Impact of Dietary Gluten on Regulatory T Cells and Th17 Cells in BALB/c Mice

Julie Christine Antvorskov[1]*, Petra Fundova[1,2], Karsten Buschard[1], David P. Funda[1,2]

1 The Bartholin Institute, Rigshospitalet, Copenhagen, Denmark, 2 Department of Immunology and Gnotobiology, Institute of Microbiology, v.v.i., Czech Academy of Sciences, Prague, Czech Republic

Abstract

Dietary gluten influences the development of type 1 diabetes (T1D) and a gluten-free (GF) diet has a protective effect on the development of T1D. Gluten may influence T1D due to its direct effect on intestinal immunity; however, these mechanisms have not been adequately studied. We studied the effect of a GF diet compared to a gluten-containing standard (STD) diet on selected T cell subsets, associated with regulatory functions as well as proinflammatory Th17 cells, in BALB/c mice. Furthermore, we assessed diet-induced changes in the expression of various T cell markers, and determined if changes were confined to intestinal or non-intestinal lymphoid compartments. The gluten-containing STD diet led to a significantly decreased proportion of $\gamma\delta$ T cells in all lymphoid compartments studied, although an increase was detected in some $\gamma\delta$ T cell subsets (CD8$^+$, CD103$^+$). Further, it decreased the proportion of CD4$^+$CD62L$^+$ T cells in Peyer's patches. Interestingly, no diet-induced changes were found among CD4$^+$Foxp3$^+$ T cells or CD3$^+$CD49b$^+$cells (NKT cells) and CD3$^-$CD49b$^+$ (NK) cells. Mice fed the STD diet showed increased proportions of CD4$^+$CD45RB^{high+} and CD103$^+$ T cells and a lower proportion of CD4$^+$CD45RB^{low+} T cells in both mucosal and non-mucosal compartments. The Th17 cell population, associated with the development of autoimmunity, was substantially increased in pancreatic lymph nodes of mice fed the STD diet. Collectively, our data indicate that dietary gluten influences multiple regulatory T cell subsets as well as Th17 cells in mucosal lymphoid tissue while fewer differences were observed in non-mucosal lymphoid compartments.

Introduction

Several studies in non-obese diabetic (NOD) mice as well as Biobreeding (BB) rats have documented that the pathogenesis of type 1 diabetes (T1D) is influenced by diet [1]. It has been demonstrated that a gluten-free (GF) diet largely prevented diabetes onset in NOD mice: the diabetes incidence was reduced from 64% to 15% [2], and a cereal-based diet promotes diabetes development [3]. Furthermore, two large human prospective cohort studies have established a connection between early infant diet containing gluten and the development of autoantibodies against the pancreatic islets. Both studies found an increased risk (≥4) of islet autoimmunity when children were exposed to gluten-containing cereals early in life [4,5]. Moreover, studies have documented an association between T1D and celiac disease (CD), which is a disease with several autoimmune features in which gluten is the triggering agent in genetic predisposed individuals [6]. It has been proposed that undiagnosed CD increases the risk of developing secondary autoimmune disorders including T1D [7]. The prevalence of CD in children with T1D has been reported to be 2–12%, and patients with CD have an earlier onset of diabetes compared to T1D patients without CD [8,9]. Thus, dietary gluten seems to be an etiological or disease-influencing factor in T1D.

Changes in intestinal permeability have been described both in spontaneous animal models of T1D [10] and in human disease [11,12]. Changes in permeability might be a direct effect of the gliadin fraction of gluten-containing cereals, because gliadin increases zonulin release, which opens intestinal tight junctions [13,14]. There is also evidence for a primary role of the intestinal immune system in the pathogenesis of T1D. Diabetogenic T cells are initially primed in the gut [15], islet-infiltrating T cells express gut-associated homing receptors [16] and mesenteric lymphocytes transfer diabetes from NOD-mice to NOD/scid-mice [17].

The role of the intestinal immune system in the pathogenesis of T1D is important, because the gut is the physiological induction site of protective immunity and is a barrier to the outer environment. Proper development of mucosal immune responses is required for induction of tolerance vs. inflammation, controlled by various subsets of T cells and dendritic cells (DC). In both human and mice, several different T cell subsets with regulatory properties (Tregs) have been showed to play a role in maintaining a tolerant state and prevent autoimmune reactions [18].

In the present study we compared the effect of a diabetes-protective GF diet to a diabetes-permissive gluten-containing STD diet, on proportions of selected T cell subsets associated with regulatory functions ($\gamma\delta$ T cells, NKT cells and Foxp3$^+$ T cells), as well as NK cells and proinflammatory Th17 cells, in fully

immunocompetent BALB/c mice. Furthermore, we studied diet-induced changes in the expression of different T cell markers (CD103, CD45RB$^{high/low}$ and CD62L) and determined if these changes were located within mucosal lymphoid tissues (Peyer's patches (PP), mesenteric (MLN), pancreatic (PLN) lymph nodes) or the non-mucosal lymphoid compartments (spleen (S), inguinal (ILN) lymph nodes).

Materials and Methods

Animals

Timed pregnant BALB/cA BomTac mice were purchased from Taconic Europe A/S, Ejby, Denmark and kept in a Specific Pathogen Free (SPF) animal facility at the Panum Institute, Copenhagen (temperature 22 ± 2 degrees, 12 h light cycle, air changed 16 times pr hour, humidity $55\pm10\%$) with free access to water and food. At day seven after birth, female pups and the female parent were assigned randomly into two groups, to receive either the STD, gluten-containing or the gluten-free (GF) diet. Twelve (six in each group) first generation female offspring were used in the study when 6 weeks old. The experiments were performed in two independent times. The animal experiments were carried out with approval from The National Animal Experimentation Board, and experiments were performed in accordance with international guidelines for the care and use of laboratory animals.

Diets

The animals received either the STD, non-purified Altromin 1310 diet, or a GF, modified Altromin diet (Altromin, Lage, Germany). Both experimental diets were nutritionally adequate with a similar level of protein, amino acids, minerals, vitamins and trace element, only the protein source differed between the diets. These two diets have been previously used at The Bartholin Institute, to study the effect of a GF diet on diabetes incidence in NOD mice [2,19]. The exact composition of the STD and the GF diet is given in [2,19]. The protein content of the GF diet and the STD were similar (22.7% vs. 22.9%). Proteins in the STD diet were derived from wheat (25%), maize, and soya, whereas the GF diet protein source was meat and soya proteins. The two diets also had the same content of amino acids, minerals, vitamins and trace elements. The weight of the mice was monitored and both groups of animals displayed similar weight distribution.

Antibodies

The following monoclonal antibodies (mAb) were purchased from BD Pharmingen: FITC-conjugated rat anti-mouse CD45RB mAb (16A; IgG2a, κ); FITC-conjugated rat anti-mouse CD49b mAb (DX5;IgM; κ); PE-conjugated rat anti-mouse CD25 mAb (3C7; IgG2b), PE-conjugated rat anti-mouse CD62L mAb (MEL-14; IgG2a, κ); PE-conjugated rat anti-mouse CD4 mAb (H129.19; IgG2a, κ), PerCP 5.5-conjugated hamster anti-mouse CD3 mAb (145-2C11; IgG1, κ), FITC-conjugated rat anti-mouse CD103 mAb (M290;IgG2a, κ), PE-conjugated hamster anti-mouse γδ T-cell receptor mAb (GL3; IgG2, κ), PerCP-Cy5.5 rat anti-mouse CD8a mAb (53-6.7; IgG2a, κ). Purchased from eBioscience were: PE-conjugated rat anti-mouse Foxp3 mAb (FJK-16s; IgG2a, κ), FITC-conjugated rat anti-mouse CD4 mAb (RM4-5; IgG2a, κ), Alexa Flour 488-conjugated rat anti-mouse IL-17A mAb (eBio17B7; IgG2a, κ).

Cell purification and flow cytometry

Mice were sacrificed and S, ILN, MLN, PP, PLN were isolated. Cells from each organ were pooled and single-cell suspensions were prepared. Surface staining was initiated with use of the relevant mAb, and cells were incubated for 1/2 hour. Intracellular staining was carried out using the Mouse Regulatory T Cell Staining Kit (eBiocience FJK-16s, Cat.No. 88-8111) following the manufacturer procedure. Fc block (CD16/CD32) was purchased from BD Pharmingen (2.4G2; IgG2b, κ) and added to reduce Fc receptor-mediated binding. The cells were analysed by flow cytometry using a FACSscan (BD Bioscience), and data were analysed with use of CellQuest software (BD Bioscience). Isotype control antibodies were used to determine the amount of non-specific binding, and propidium iodide was used to exclude or to localize dead cells.

Statistical analysis

Student's unpaired t–test was used to compare the frequency of the cells (means $\pm$ SEM) of the two groups (STD vs. GF) of mice, and a value of $P<0.05$ was considered statistically significant. Statistical analysis and graphic presentation of the results were performed using the Sigma Plot 9.0 Software and GraphPad Prism version 5.

Results

No diet-induced difference was found in the proportion of CD4$^+$Foxp3$^+$ T cells

Cell suspensions were prepared from isolated mucosal (MLN, PP, PLN) and non-mucosal (S, ILN) lymphoid organs and stained for CD3, CD4 and Foxp3 markers, and percentages of CD4$^+$Foxp3$^+$ T cells was assessed within each of the studied lymphoid tissues (gating shown in Fig. 1A). No significant difference in the proportion of CD4$^+$Foxp3$^+$ T cells was found in the mucosal lymphoid tissues: PLN (11.6 ± 0.11 vs. $11.5\pm0.06\%$, P = 0.27), MLN (15.1 ± 1.99 vs. $11.9\pm1.14\%$, P = 0.29) and PP (14.5 ± 0.10 vs. $14.2\pm0.19\%$, P = 0.36) in the BALB/c mice fed the STD compared to the GF diet. Similarly, no significant difference in percentages of CD4$^+$Foxp3$^+$ T cells was found in S (18.9 ± 0.60 vs. $17.1\pm0.52\%$, P = 0.14) and ILN (11.5 ± 0.34 vs. $11.9\pm0.55\%$, P = 0.59) between the two groups of animals (Fig. 1B). These results were confirmed by a CD3$^+$CD4$^+$CD25$^+$ staining and analysis for CD4$^+$CD25^{high} T cells (data not shown). Thus, we found no diet-induced differences in the proportion of CD4$^+$Foxp3$^+$ T cells, neither in mucosal lymphoid organs (MLN, PP, PLN) nor in non-mucosal lymphoid compartments (S, ILN).

The STD diet induced a significant increase in CD4$^+$IL-17$^+$ (Th17) cells in PLN

Th17 cells, a population of CD4$^+$ T helper cells that secretes a set of proinflammatory cytokines including IL-17, is considered a distinct cell lineage that seems to antagonize Treg development and thus promote autoimmunity [20–22]. To determine gluten-induced changes in the Th17 cell subset, we first gated according to the forward and side scatter parameters, followed by CD3 gating and percentages of CD4$^+$IL-17$^+$ cells were measured (Fig. 2 B/C). Significantly (P<0.05) higher proportion of CD4$^+$ IL-17$^+$ T cells was found only in PLN of BALB/c mice on the STD diet ($1.4\pm0.05\%$) compared to mice fed the GF diet ($0.7\pm0.14\%$)(Fig. 2A). The proportion of CD4$^+$IL-17$^+$ T cells in all other organs (S, ILN, MLN, PP) showed no diet-induced differences. Thus, the gluten-containing STD diet induced significantly increased proportion of Th17 cells in lymph nodes associated with pancreatic tissue (Fig. 2A/C).
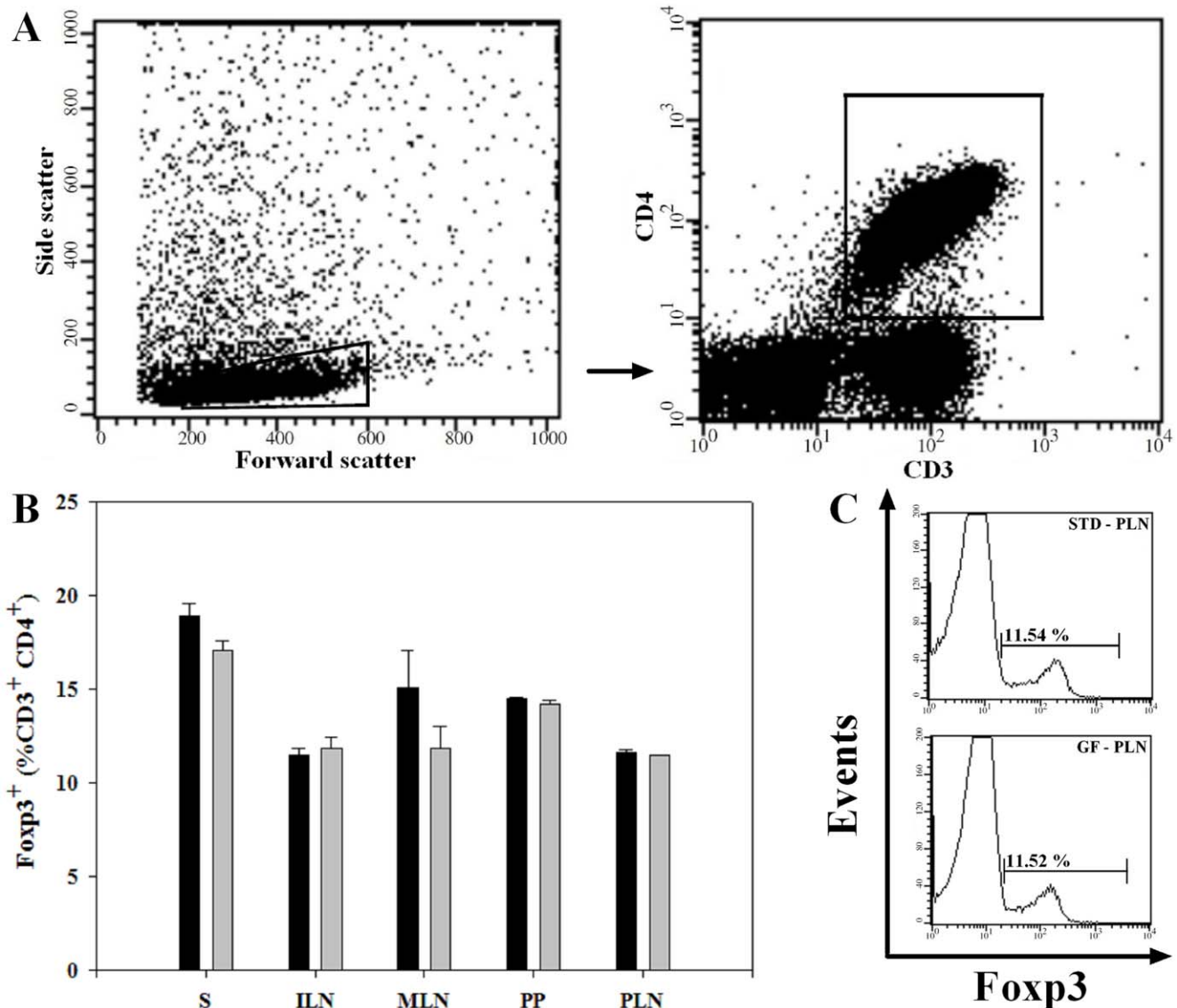
Figure 1. No diet-induced difference was found in the proportion of CD4+Foxp3+ T cells. (A) Representative plot of lymphocyte and CD3+CD4+ gate. (**B**) Bars represent the proportion of Foxp3+ cells (gated on 100% CD4+CD3+ cells) in various lymphoid compartments. No diet-induced differences was found in the proportion of CD4+Foxp3+ T cells in all lymphoid compartments tested. Data are presented as mean values ± SEM of two independent experiments with 6 mice in each group. (**C**) Representative histogram from PLN, showing the proportion of CD4+Foxp3+T cells in the STD diet mice compared to the GF diet mice. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN). Black bars: STD diet. Grey bars: GF diet.
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The STD diet reduced the proportion of γδ T cells

Changes in the γδ T cell subset have been documented in NOD mice [23] as well as in human T1D [24–26], In addition γδ T cells have been shown to play an important role in induction of mucosal tolerance and prevention of T1D in the NOD mouse [27,28]. We found a significantly lower proportion of γδ T cells in all tested lymphoid tissues in BALB/c mice fed the STD diet compared to mice receiving the GF diet, S (1.3±0.06 vs. 2.0±0.17%, P<0.05), ILN (1.0±0.01 vs. 1.3±0.03%, P<0.01), MLN (1.6±0.05 vs. 2.0±0.05%, P<0.05), PP (1.4±0.02 vs. 1.9±0.03%, P<0.01) and in PLN (1.0±0.005 vs. 1.8±0.03%, P<0.001) (Fig. 3A). Thus, the gluten-containing STD diet reduces the overall proportion of γδ T cells in both mucosal lymphoid organs (MLN, PP, PLN) and in non-mucosal lymphoid compartments (S, ILN). Next, we analysed the expression of CD8 and the mucosal homing marker CD103

within the γδ T cell subset (Fig. 3B) and observed reciprocal differences: in mice fed the STD diet there is an overall increase in γδ T cells expressing CD103, with significantly higher proportion in MLN (34.1±0.66 vs. 11.5±1.12%, P<0.01) and in PLN (34.7±1.05 vs. 15.6±1.36%, P<0.01) compared to mice receiving the GF diet (Fig. 3C). The same pattern was found when evaluating the co-expression of CD8+ and CD103+ on γδ T cells: the proportion of CD8+CD103+ γδ T cells in MLN (16.3±2.36 vs. 5.7±1.11%, P<0.05) and PLN (18.8±0.34 vs. 7.7±0.38%, P<0.01) was higher in mice receiving the STD diet compared to the GF diet (Fig. 3D). Finally, the expression of CD8 within γδ T cells was higher in PLN (37.0±3.18 vs. 18.3±0.06%, P<0.05) in mice on the STD diet vs. the GF diet (Fig. 3E). Consequently, a reversed pattern was observed in the remaining subset of CD8−CD103− γδ T cells. Thus MLN (55.5±0.98 vs.
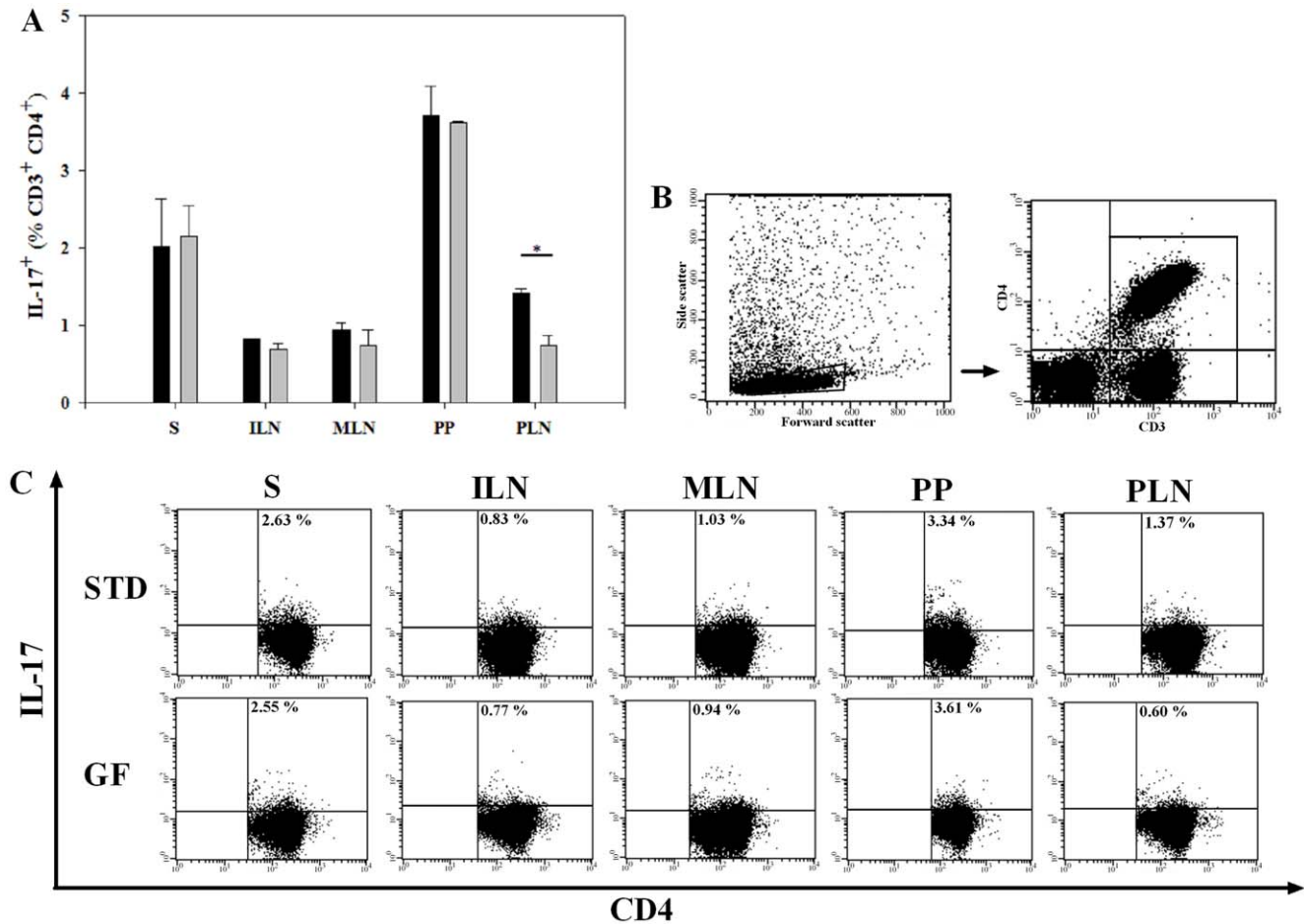
Figure 2. The STD diet induced a significant increase in CD4⁺IL-17⁺ (Th17) cells in PLN. (A) Bars represent percentages of IL-17⁺ cells gated on 100%CD3⁺CD4⁺ cells. BALB/c mice on the STD diet showed an increased proportion of CD4⁺IL-17⁺ T cells in PLN, compared to mice on the GF diet. Data are presented as mean values $\pm$ SEM of two independent experiments with 6 mice in each group. (**B**) Representative example of the lymphocyte gate and CD3⁺ gate. (**C**) Examples of dot plots showing the percentages of IL-17⁺ T cells, gated on 100% CD3⁺CD4⁺ cells. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN). Black bars: STD diet. Grey bars: GF diet. * P<0.05.
doi:10.1371/journal.pone.0033315.g002

74.5±0.44%, P<0.01) and PLN (47.1±1.1.79 vs. 73.7±1.04%, P<0.01) of STD diet fed BALB/c mice revealed a significantly decreased proportion of CD8⁻CD103⁻ γδ T cells (Fig. 3F). In summary, there is a lower proportion of γδ T cells both in non-mucosal (S, ILN) and mucosal lymphoid organs (MLN, PP, PLN) but a higher proportion of CD8⁺γδ T cells where the majority express the mucosal homing marker CD103 in mucosal lymphoid organs (MLN, PLN) in BALB/c mice receiving the STD diet, than in mice receiving the GF diet.

The proportion of CD4⁺T cells expressing CD45RB^high and CD45RB^low was influenced by the diet

The CD45RB antigen is highly expressed on various subsets of lymphocytes. The expression of CD45RB on CD4⁺ T cells distinguishes naïve (CD45RB^high) from activated or memory (CD45RB^low) cells. Furthermore, the expression of CD45RB is associated with distinct immunological functions of the T cell [29,30]. We found that BALB/c mice on the STD diet displayed a significant lower proportion of CD4⁺CD45RB^low T cells in S (73.9±0.35 vs. 82.8±0.40%, P<0.01) and in PP (86.6±0.49 vs. 90.2±0.61%, P<0.05), than mice on the GF diet (Fig. 4A). The reciprocal CD4⁺CD45RB^high T cell population was increased in

mice on the STD compared to the GF diet, with a significant increase in PP (4.9±0.01 vs. 4.5±0.05%, P<0.01) and PLN (2.2±0.05 vs. 1.4±0.03%, P<0.01) (Fig. 4B). A representative example of the CD45RB FACS analysis and the differences in the proportion of CD45RB expression in PLN is shown in Fig. 4C. In conclusion, mice receiving the gluten-containing STD diet displayed a significantly lower proportion of CD4⁺CD45RB^low T cells in S and PP, and a higher percentage of CD4⁺CD45RB^high T cells in PP and PLN.

Fewer CD4⁺ T cells in PP expressed CD62L in mice receiving the STD diet

CD62L (L-selectin) is a member of the selectin adhesion molecule family, and is required for leukocyte entry from circulation into secondary lymphoid tissues such as peripheral lymph nodes [31]. When studying an effect of the STD gluten-containing diet on CD62L expression within the CD4+ T cells (Fig. 5), we found no substantial differences in non-mucosal lymphoid compartments (S, ILN) as well as in MLN and PLN associated with the mucosal lymphoid tissue. However, there were significantly fewer CD4⁺CD62L⁺ T cells in mice receiving the STD diet compared to the GF diet in PP (32.9±0.26 vs.
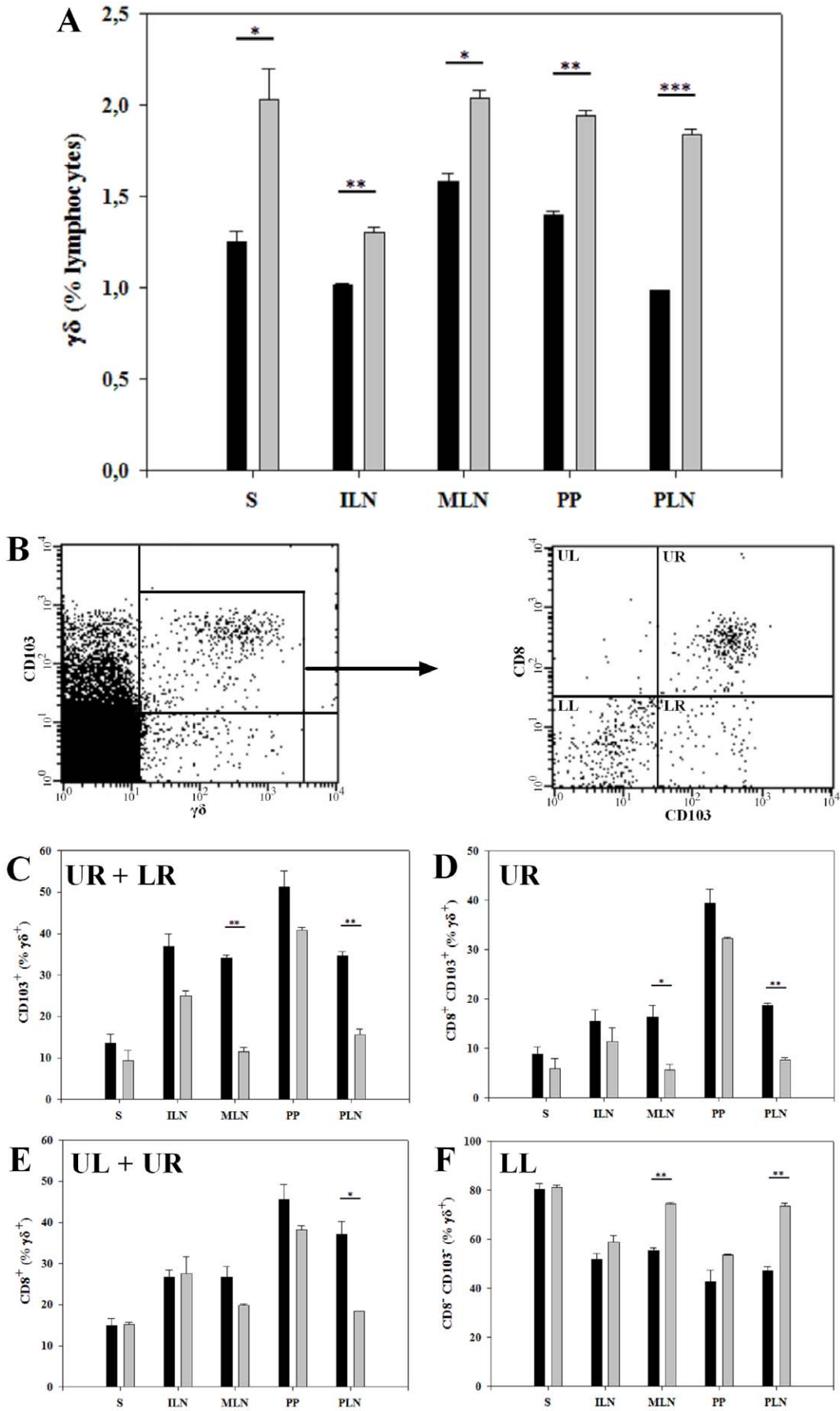
Figure 3. The STD diet reduced the proportion of γδ T cells. (**A**) Bars represent percentages of γδ T cells. The proportion of γδ T cells was reduced in BALB/c mice on the gluten-containing STD diet in all tested lymphoid compartments. (**B**) Representative dot plots illustrating gating of γδ T cells, and the subsequent analysis of subpopulation of γδ T cells, according to CD8 and CD103 expression. (**C**) Percentages of γδ T cells expressing CD103. Gluten induced a significant increase in CD103 expression on γδ T cells in MLN and PLN. (**D**) Percentages of CD8⁺CD103⁺ cells gated on 100% γδ⁺ cells, with an increased proportion in MLN and PLN in mice on the STD compared to the GF diet. (**E**) Percentages of γδ T cells expressing CD8. The proportion of CD8⁺ γδ T cells was higher in PLN in mice receiving the STD compared to the GF diet. (**F**) Percentages of CD8⁻CD103⁻ γδ T cells was lower in mice on the STD than on the GF diet, with a significant decrease in MLN and PLN. Barplots are mean values ± SEM of two independent experiments with 6 mice in each group. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN). Black bars: STD diet. Grey bars: GF diet. * P<0.05. ** P<0.01. *** P<0.001.
doi:10.1371/journal.pone.0033315.g003

42.9±2.32%, P<0.05)(Fig. 5A). A representative example of isolated PP is shown in Fig. 5B. Although not significant, a decreased percentage of CD62L⁺CD4⁺ T cells was also noticed in PLN in mice receiving the STD diet. In a representative example we found 76.57% compared to 83.44%. In conclusion, there were fewer CD4⁺ T cells expressing the CD62L homing marker in PP of BALB/c mice fed the STD diet than in mice on the GF diet but no differences were found in CD62L expression on

CD4⁺ T cells in all other lymphoid compartments (S, ILN, MLN, PLN).

Increase in CD103⁺ T cells in S and PLN in mice receiving the STD diet

The integrin CD103 (αEβ7 integrin) is responsible for an efficient homing and retention of lymphocytes to gut-associated lymphoid tissue, gut epithelium and lamina propria [32]. To
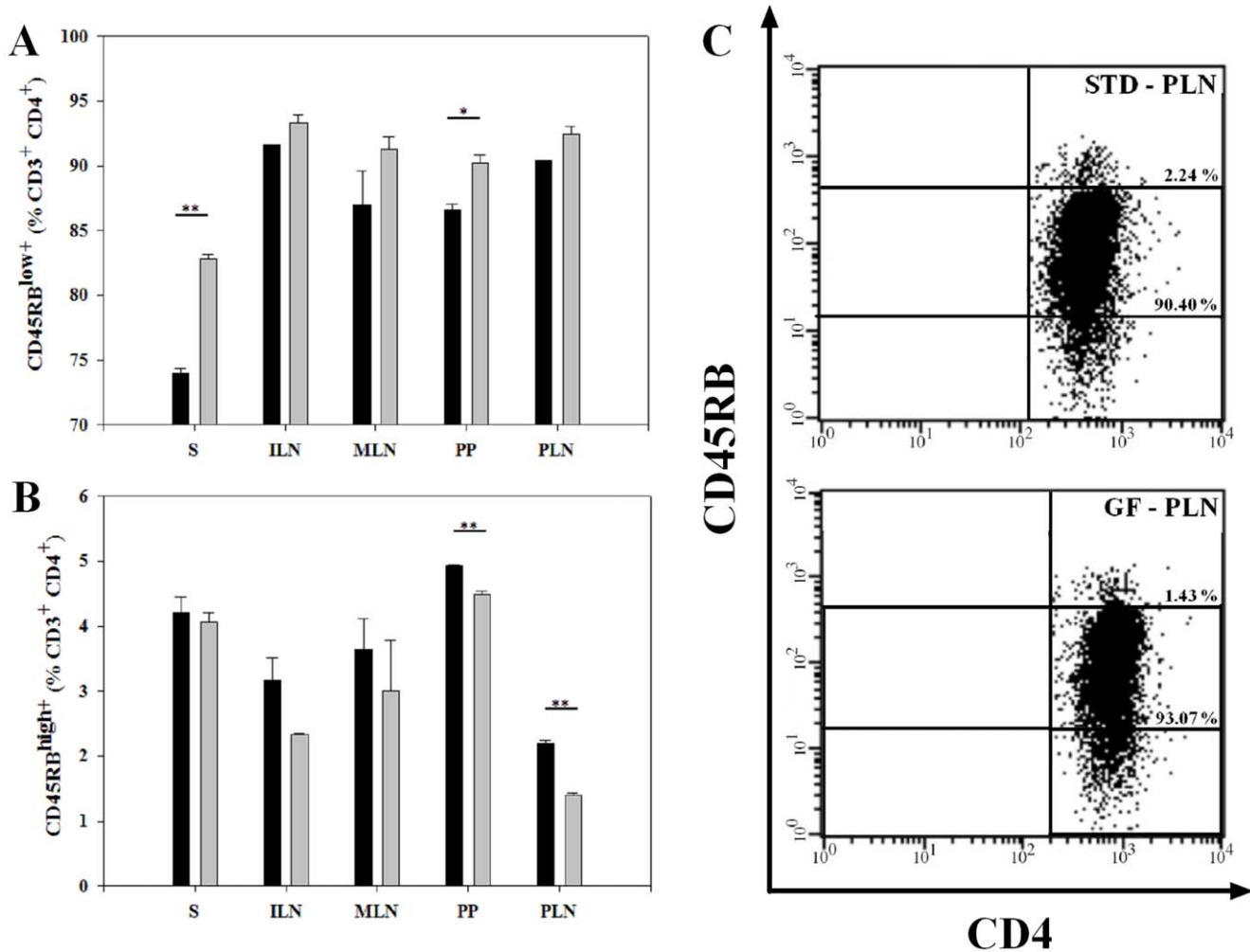

Figure 4. The proportion of CD4⁺T cells expressing CD45RBhigh and CD45RBlow was influenced by the diet. (**A**) Bars represent percentages of CD45RBlow expressing cells gated on 100% CD3⁺CD4⁺ cells. There was a decreased proportion of CD4⁺ CD45RBlow T cells in S and PP of BALB/c mice receiving the STD diet compared to the GF diet. (**B**) Percentages of cells expressing CD45RBhigh gated on 100% CD3⁺CD4⁺ cells. There was an increased proportion of CD4+CD45RBhigh T cells in PP and PLN in mice receiving the STD diet. Data are presented as mean values ± SEM of two independent experiments with 6 mice in each group. (**C**) Representative dot plot of cells isolated from PLN showing the proportion of CD45RBlow and CD45RBhigh CD4⁺ T cells from STD mice vs. GF mice. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN). Black bars: STD diet. Grey bars: GF diet. * P<0.05. ** P<0.01.
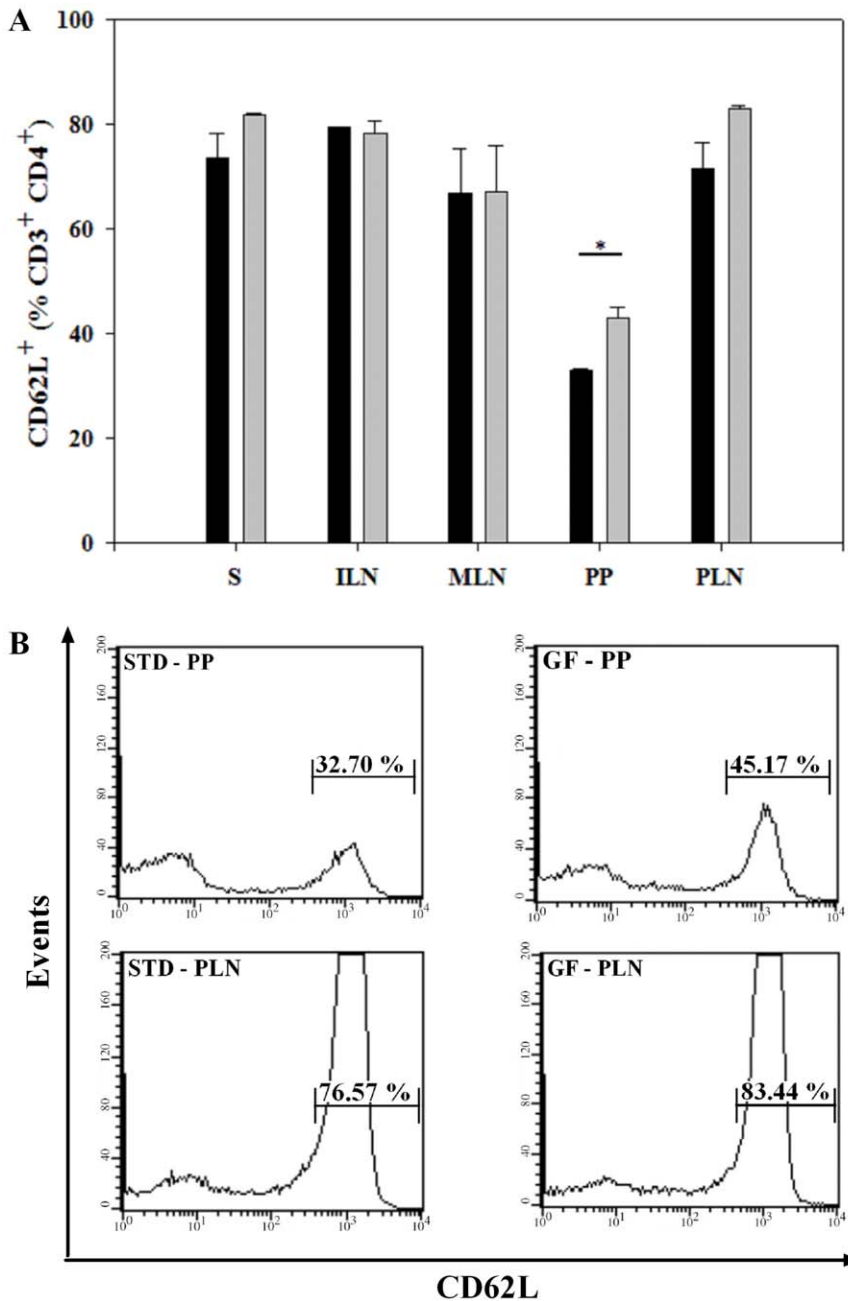doi:10.1371/journal.pone.0033315.g004

Figure 5. Fewer CD4$^+$ T cells in PP expressed CD62L in mice receiving the STD diet. (**A**) Bars represent percentages of CD62L$^+$ cells gated on 100% CD3$^+$CD4$^+$ cells. In BALB/C mice receiving the STD diet, compared to mice receiving the GF diet, there was a decreased proportion of CD4$^+$CD62L$^+$ T cells in PP. Data are presented as mean values ± SEM of two independent experiments with 6 mice in each group. (**B**) Representative histograms from PP and PLN showing the differences in CD4$^+$CD62L$^+$ T cells. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN).Black bars: STD diet. Grey bars: GF diet. * P<0.05.
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determine diet-induced differences in CD103 expression on T cells, we first assessed differences within the CD3$^+$ gated cells. We found a significantly higher proportion of CD103$^+$ T cells in mice on the STD diet vs. the GF diet, in S (15.4±0.11 vs. 13.8±0.06%, P<0.01) and in PLN (13.8±0.20 vs. 9.21±0.70%, P<0.05) (Fig. 6A). A representative staining of CD3$^+$CD103$^+$ cells isolated from PLN is shown in Fig. 6B. However, when gating on CD3$^+$CD4$^+$ T cells we found no significant diet-induced differences in the CD103 expression (Fig. 6C/D). CD103 positive T cells are thus found to be

expanded in S and PLN of BALB/c mice receiving the STD diet, compared to the GF diet, but this pattern is not present within the CD4$^+$ T cells.

No diet-induced difference was found in the proportion of CD49b$^+$ T cells and CD49b$^+$ non-T cells

Because BALC/c mice do not express the NK1.1 marker, we have used anti-CD49b (clone DX5) pan-NK mAb that was shown to almost entirely overlap with the NK1.1 staining in C57Bl/6 mice as an NK cell marker [33]. We assessed its expression within
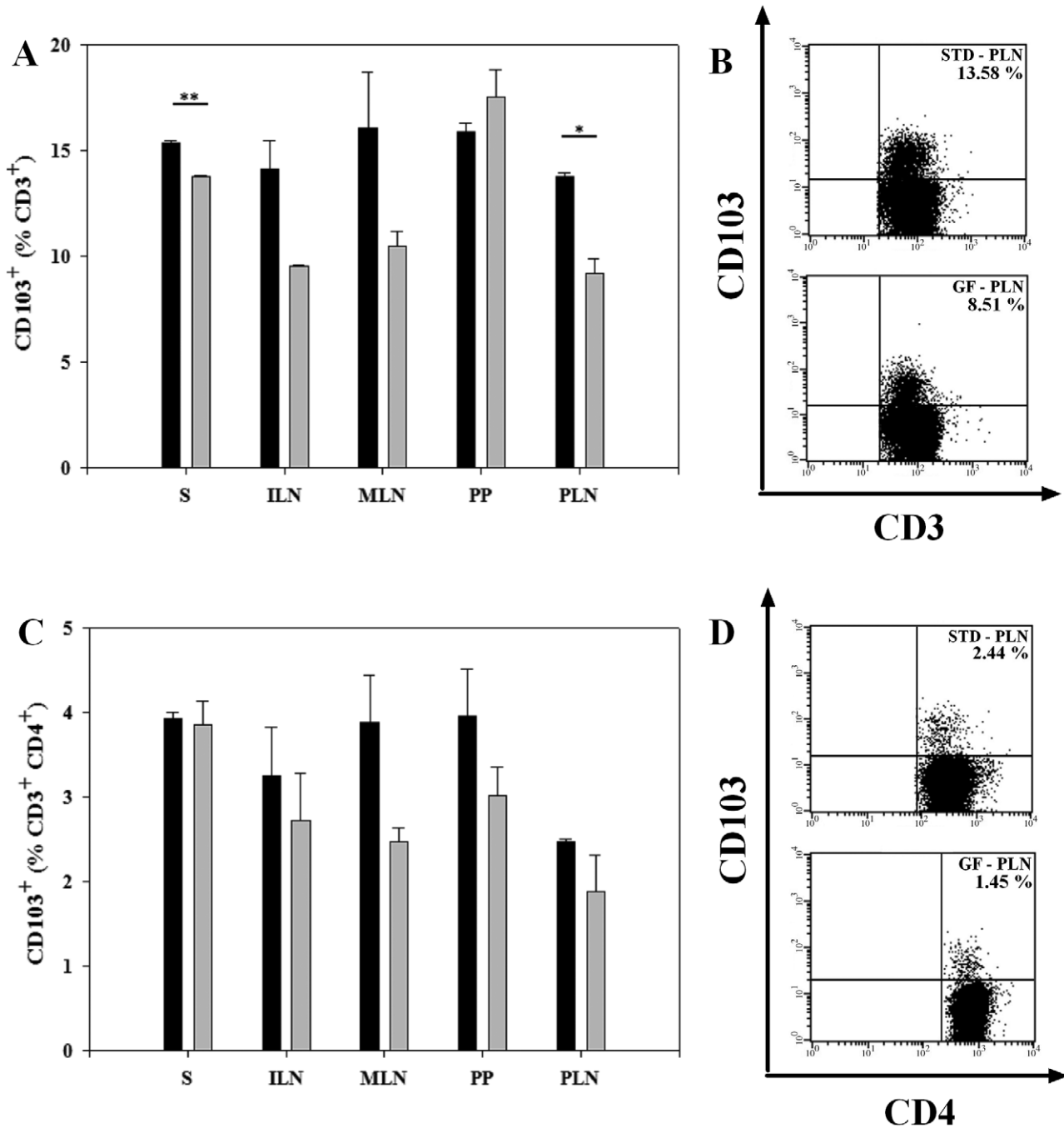
Figure 6. Increase in CD103$^+$ T cells in S and PLN in mice receiving the STD diet. (**A**) Bars represent the proportion of CD103$^+$ cells, gated on 100% CD3$^+$ cells. There was an increased proportion of CD103 expression in S and PLN in BALB/c mice receiving the STD diet. (**B**) Representative dot plot from PLN showing the differences in CD103$^+$ T cells between the two diets. (**C**) Bars showing the percentages of CD103$^+$ cells gated on 100% CD3$^+$CD4$^+$ lymphocytes. (**D**) Representative dot plots of CD4$^+$CD103$^+$ T cells from isolated PLN. Barplots are mean values $\pm$ SEM of two independent experiments with 6 mice in each group. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN). Black bars: STD diet. Grey bars: GF diet. * P<0.05. ** P<0.01.
doi:10.1371/journal.pone.0033315.g006

the CD3$^+$ and the CD3$^-$ cells. No statistically significant difference in proportions of CD3$^+$CD49b$^+$ or CD3$^-$CD49b$^+$ cells were found in neither S, ILN, MLN, PP, PLN of BALB/c mice fed the STD versus GF diets (Fig. 7A/B). Although not significant a tendency towards a lower level of CD3$^+$CD49b$^+$ (NKT cells) were found in mice fed the STD versus the GF diet (Fig. 7A).

Discussion

T1D is an autoimmune disease that is characterized by dysregulated T cell activation and diminished regulatory T cell function [34,35]. Gluten intake has been associated with the development of T1D in both animal models of T1D [1,3] and in humans [4,5], but the effect of dietary gluten on T cell subsets
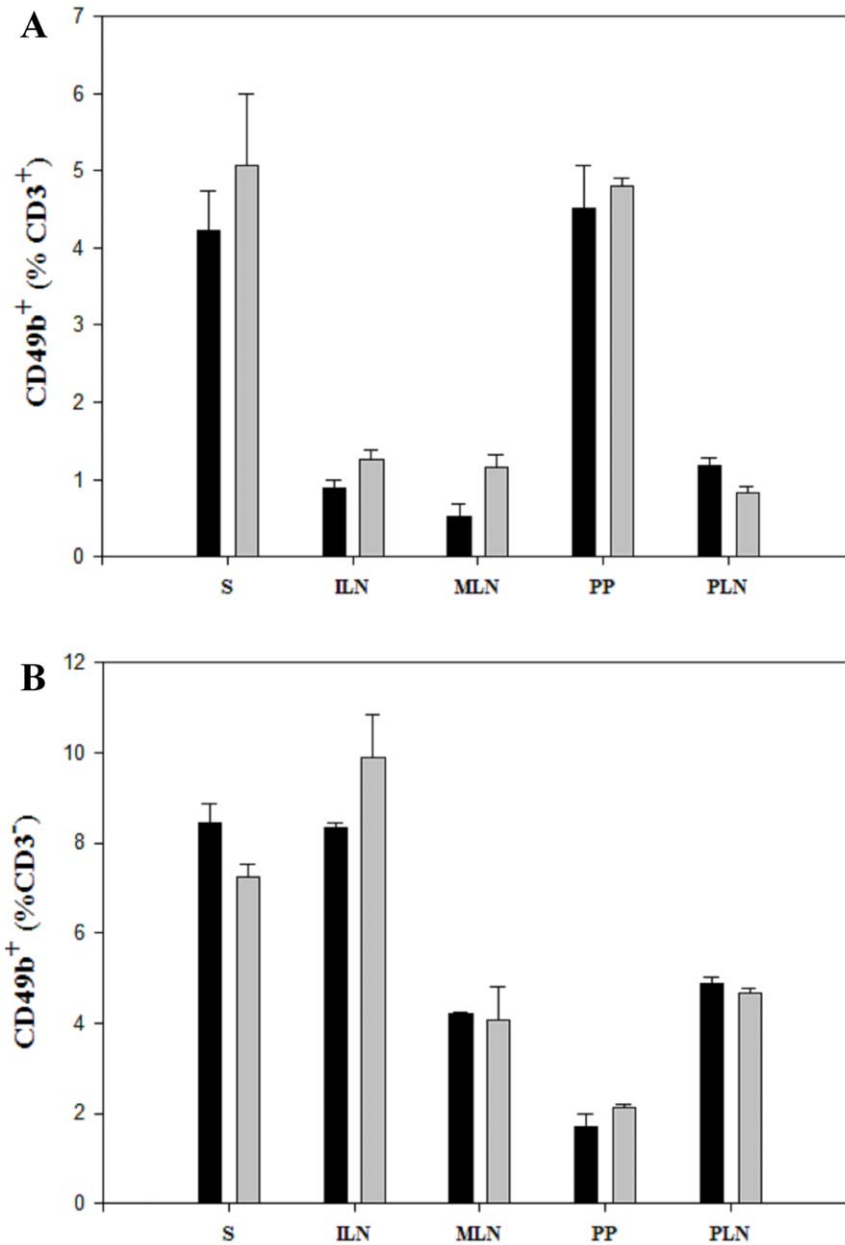
Figure 7. No diet-induced difference was found in the proportion of CD3⁺CD49b⁺ (NKT cells) or CD3⁻CD49b⁺ (NK cells). (**A**) Bars represent the proportion of CD49b⁺ cells, gated on 100% CD3⁺ cells. No diet induced differences was found. (**B**) Bars showing the percentages of CD3⁻CD49b⁺ cells, with no significant differences between the two diets. Barplots are mean values ± SEM of two independent experiments with 6 mice in each group. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN). Black bars: STD diet. Grey bars: GF diet.
doi:10.1371/journal.pone.0033315.g007

associated with regulatory function has not been well characterized. In order to assess the effect of dietary gluten on various T cell subsets, experiments were carried out in healthy immunocompetent BALB/c mice, as NOD mice are known to be lymphophenic [28] as well as they display other immune alterations [36].

Collectively, our data show that a gluten-containing STD diet decreases the proportion of γδ T cells in all lymphoid compartments studied, but reciprocal differences was detected in CD8⁺ and CD103⁺ γδ T cells in mucosal lymphoid compartments. This is the most consistent change among the T cell subsets studied according to gluten influenced changes. However we also

found substantially decreased proportion of CD62L⁺CD4⁺ T cells in PP and of CD4⁺CD45RB^low+ T cells in S and PP associated with the STD diet. Furthermore, mice fed the STD diet showed increased proportion of CD4⁺CD45RB^high+ in PP and PLN and of CD103⁺ T cells in S and PLN. Interestingly, no diet-induced changes were found in CD4⁺Foxp3⁺ T cells, CD3⁺CD49b⁺ (NKT cells) or CD3⁻CD49b⁺ (NK cells). The Th17 cell population, associated with the development of autoimmunity [37,38], was substantially increased in PLN of mice fed the STD diet. A summary of the changes in T cells (CD3⁺) subsets and cellular markers in BALB/c mice receiving the gluten-containing STD diet compared to the GF diet is given in Fig. 8.
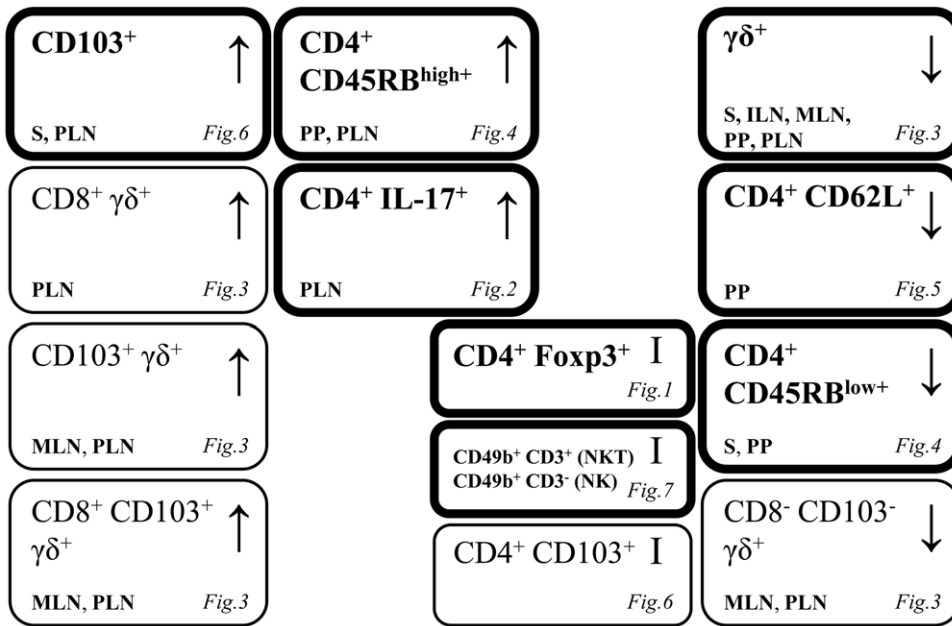
Figure 8. Summary of changes in T cells (CD3$^+$) subsets and cellular markers in BALB/c mice receiving the gluten-containing STD diet compared to the GF diet. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN). Significant diet-induced changes within specific lymphoid compartment are indicated in each box. ↑: Increased proportion in animals receiving the gluten-containing STD diet. ↓: Decreased proportion in animals receiving the gluten-containing STD diet. I: No diet-induced changes. Boxes with thin line: Subsets of T cells expressing a specific cellular marker e.g. γδ T cells expressing CD103.
doi:10.1371/journal.pone.0033315.g008

Evidence derived from both human and animal studies suggests that a deficiency in either the frequency or the function of Tregs could be associated with T1D development [34]. Foxp3-expressing Tregs are a constitutively occurring T cell subpopulation, whose deficiency is associated with development of autoimmunity, and which controls adaptive immune responses both in health and disease [39]. We found no diet-induced changes in the proportion of Foxp3$^+$ Tregs in neither mucosal lymphoid tissue (MLN, PP, PLN) nor in non-mucosal lymphoid compartments (S, ILN). Previously, one study reported that gluten intake reduces the proportion of Foxp3$^+$ T cells in PP of BALB/c mice [40]. The discrepancy between the studies could be due to differences in the age at which the BALB/c mice were introduced to the gluten-free diet or whether the mice were exposed to gluten during lactation. Other studies do, however, find that the proportion of Foxp3$^+$ Tregs is not influenced by gluten intake as in human CD patients [41] or in T1D patients or in individuals at varying degrees of risk for T1D [42]. Thus it seems that the frequency of Foxp3$^+$ Tregs is not related to disease development or prevention but a defective regulatory function in Foxp3$^+$ Tregs could still play a role in susceptibility to the disease. This view is supported by studies showing defective suppressor function in CD4$^+$CD25$^+$ T cells from T1D patients [43,44] and by studies showing that Foxp3 mRNA expression (relative to the number of Foxp3$^+$ Tregs) is reduced in patients with potential CD or T1D [45,46]. The authors suggest that this could indicate that the maintenance of oral tolerance to gluten is not related to a higher proportion of Foxp3$^+$ T cells, but is related to the up-regulation of Foxp3 transcripts [46]. Therefore it seems that functional changes in Foxp3$^+$ Tregs, rather than their frequency might be involved in the susceptibility for developing T1D.

Interestingly, we found that the gluten-containing STD diet specifically raised the proportion of Th17 cell in PLN. The Th17 subsets are considered to be a key mediator of distinct autoimmune diseases, as rheumatoid arthritis [47], multiple sclerosis [48], inflammatory bowel disease [49], and CD [50,51]. Recently Th17 cells have been described to be involved in T1D, both in animal models [38] and in humans [37]. The exact role of Th17 cells in the diabetogenic process remains to be fully determined. However, it has been shown that the insulitis lesions in the NOD mice contain high levels of IL-17 transcript [52]. Moreover, inhibition of Th17 cells with neutralising anti-IL-17 antibodies or with recombinant IL-25 prevents diabetes in NOD mice when treatment is started at 10 weeks but not 5 weeks of age [53], illustrating the importance of this cell population in the pathogenesis of T1D as well as the role of different time windows required for T1D preventive interventions. To our knowledge, gluten-induced Th17 priming in pancreatic lymph nodes has not previously been described. This finding thus points towards possible mechanisms by which dietary gluten may have an etiological role in T1D.

Several lines of evidence suggest a role for γδ T cells in T1D [24–26]. Their role in the pathogenesis seems to be complex, with indications of their involvement both in regulatory and pathogenic pathways. NOD mice display an increased proportion of γδ T cells at onset of diabetes [23], and in BB rats the development of γδ T cells is inhibited due to the *lyp* mutation, which is a marker of susceptibility to diabetes [54]. Recently, insulin-specific γδ T cells were found in NOD mice, and the authors suggest that these insulin-specific γδ T cells are involved in the auto-immune response leading to T1D [55]. We found a significantly lower proportion of γδ T cells both in non-mucosal and mucosal lymphoid organs in mice receiving the STD diet, compared to the GF diet. This is the most consistent change detected among the T cell subsets, in respect to the two diets tested. However, a higher proportion of CD8$^+$ γδ T cells and γδ T cells expressing the mucosal homing marker CD103 were found in mice on the STD diet vs. the GF diet. Although, the exact role of γδ T cells is

remains to be further studied, several findings have documented an importance of γδ T cells in induction of tolerance. Thus, nasal exposure to insulin induces CD8⁺γδ T cells capable of suppressing diabetes development in mice [27], anti-γδ antibody disrupts oral mucosal tolerance to ovalbumin in mice [56], and oral administration of antigen does not induce tolerance in TCRδ-knockout mice [57]. Moreover, it have been shown that intraepithelial CD8⁺γδ T cells prevent diabetes, and intraepithelial γδ T cells are required for induction of regulatory CD4⁺CD25⁺ T cells by oral insulin, in the neonatal thymectomy NOD mouse model of T1D [28]. These data suggest that oral tolerance to antigens is induced and actively maintained by mechanisms involving γδ T cells. Overall, the intake of dietary gluten reduces the proportion of γδ T cells in both non-mucosal and mucosal lymphoid organs, but it increases the proportion of potentially regulatory CD8⁺ γδ T cells expressing the intestinal homing marker CD103 in mucosal lymphoid organs. To support these findings, we determined diet-induced differences in the expression of CD103. We found an increase in the proportion of CD103⁺ T cells in S and PLN of BALB/c mice receiving the gluten-containing STD diet. The CD103 integrin (αEβ7 integrin) is important for homing of T cells to the intestinal compartment, as it mediates adhesion to epithelial cells due to its binding to E-cadhedrin, expressed selectively on epithelial cells [58]. Our data indicate that gluten leads to an immune activation within the intestinal compartment that causes upregulation of CD103 on lymphocytes.

Several T cell subsets with regulatory properties have been demonstrated to prevent diabetes development in the NOD mouse model. Apart from the regulatory role of γδ T cells in T1D [27,28] CD4⁺CD62L⁺ T cells have been shown to inhibit development of the disease in NOD mice [59,60] Here we show a decreased proportion of CD4⁺CD62L⁺ T cells in PP and a similar tendency in PLN of BALB/c mice fed the gluten-containing STD diet compared to the GF diet. Since expression of CD62L is critical for leukocyte trafficking into secondary lymphoid organs [31], the diabetes-permissive STD diet may be associated with lower proportion of naïve CD4⁺ T cells migrating to mucosal compartments such as PP or the PLN. The increased proportion of CD62L expression in PP and a similar tendency in PLN in mice fed the diabetes-preventive GF diet is also interesting in respect to the observation that Tregs expressing high levels of CD62L posses stronger immunosuppressive potential [61].

Transfer of CD4⁺CD45RB^high expressing T cells into an immunodeficient mouse induces chronic intestinal inflammation [62] and this population of cells is conventionally used to induce inflammatory bowel disease in mice [63], illustrating their

pathogenic potential. Co-transfer of lymphocytes expressing CD4⁺CD45RB^low, on the other hand, prevents disease development [64]. Gluten-induced changes in CD45RB expression on CD4⁺ T cells could be important for T1D development since subpopulations of CD4⁺ T cells expressing high or low levels of CD45RB, have different cytokine profiles and mediate distinct immunological functions [65]. CD45RB^low T cells were reported to have a regulatory cytokine profile with IL-10 and IL-4 production [30], opposite to CD45RB^high expressing cells, producing more proinflammatory cytokines as IFNγ [29] and TNFα [30]. We found an increase of CD4⁺CD45RB^high T cells in PP and PLN, while the CD4⁺CD45RB^low T cell population was decreased in S and PP, in mice fed the STD diet. Gluten may therefore increases a subset of CD4⁺ T cells, expressing CD45RB^high, that may induce inflammation in the intestine, perhaps due to their secretion of proinflammatory cytokines. In this respect, it is interesting that the proportion of CD4⁺CD45RB^high T cells was increased only in mucosal compartments (PP, PLN).

In summary, our present study reveals that the gluten-containing STD diet, that allows high diabetes penetrance in spontaneous, animal models of T1D, significantly decreases the proportion of different potentially regulatory T cell populations, especially γδ T cells and CD4⁺CD62L⁺, but not CD4+Foxp3⁺ T cells. Moreover, the STD diet modifies the proportions of CD4⁺CD45RB^low+ and CD4⁺CD45RB^high+ T cells and leads to an increased proportion of proinflammatory Th17 cells in pancreas draining lymph nodes. Interestingly, many of the described changes were localized specifically within the mucosal compartment (MLN, PP) and PLN. Thus, our data show the effect of dietary gluten on the gut-associated immune system and the potential, etiological role of gluten in T1D. We suggest that failure to develop proper regulatory immune responses to environmental stimuli, such as dietary gluten, may lead to a higher incidence of T1D in genetically predisposed individuals.

Acknowledgments

Author Contributions

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3.2.2. Publication V.

This study is a follow-up of previous paper. In animal models of T1D, the dietary prevention of the disease is well documented, but the mechanisms behind the diet-mediated modification of diabetes incidence are not well understood. A cytokine shift towards Th1 profile was described in the gut of NOD mice fed diabetes-permissive standard diet (Flohe et al., 2003). In BB rats a shift from a Th1 to Th2 cytokine pattern has been described in the islet infiltrate of BB rats fed a hydrolyzed casein diet (Scott et al., 1997).

In this study we investigated cytokine profiles of polyclonally (PMA + ionomycin) stimulated Foxp3$^+$ Tregs as well as Foxp3$^-$ T cells from mucosal (MLN, PLN, PP) and non-mucosal (spleen, ILN) lymphoid organs from immunocompetent BALB/c mice that were fed neonatelly a standard, gluten-containing, diabetes permissive diet compared to litters fed gluten-free, diabetes preventive diet.

Within CD4$^+$ T cells the gluten-containing, standard diet increased numbers of IFN-γ, IL-17 and IL-2 producing cells in all organs studied. In Foxp3+ Tregs, there was a shift towards more IL-2, and IL-17 (in mucosal organs) as well as IL-4 and IFN- γ positive cells (in both compartments). On the other hand, the gluten-free diet was associated with increased proportion of TGF-β positive CD4$^+$ T cells. The expression of IFN-γ in Tregs was a surprising finding, that was, however corresponding with the plasticity of Tregs published J. Bloostone group (Zhou et al., 2009). Collectively, our results document that the gluten-containing, diabetes permissive diet is causing a more proinflammatory cytokine signature in both CD4$^+$ T cells as well as Foxp3$^+$ Tregs. We think the proinflammatory properties of the diabetes permissive, gluten-based diet points towards a hypothesis, that a higher gluten intake could be associated with an increased diabetes incidence.

IMMUNOLOGY ORIGINAL ARTICLE

Dietary gluten alters the balance of pro-inflammatory and anti-inflammatory cytokines in T cells of BALB/c mice

Julie C. Antvorskov,[1] Petra Fundova,[1,2,3] Karsten Buschard[1] and David P. Funda[1,3]

[1]*The Bartholin Institute, Rigshospitalet, Copenhagen, Denmark,* [2]*ENT Department of the 3rd Faculty of Medicine, Charles University and the Central Military Hospital, Prague, and* [3]*Department of Immunology & Gnotobiology, Institute of Microbiology, v.v.i., Czech Acad. Sci., Prague, Czech Republic*

Summary

Several studies have documented that dietary modifications influence the development of type 1 diabetes. However, little is known about the interplay of dietary components and the penetration of diabetes incidence. In this study we tested if wheat gluten is able to induce differences in the cytokine pattern of Foxp3^{+} regulatory T cells, as well as Foxp3^{−} T cells, isolated from intestinal mucosal lymphoid tissue and non-mucosal lymphoid compartments in BALB/c mice. The gluten-containing standard diet markedly changed the cytokine expression within Foxp3^{−} T cells, in all lymphoid organs tested, towards a higher expression of pro-inflammatory interferon-γ (IFN-γ), interleukin-17 (IL-17) and IL-2. In Foxp3^{+} regulatory T cells, gluten ingestion resulted in a mucosal increase in IL-17 and IL-2 and an overall increase in IFN-γ and IL-4. The gluten-free diet induced an anti-inflammatory cytokine profile with higher proportion of transforming growth factor-β (TGF-β)[+] Foxp3^{−} T cells in all tested lymphoid tissues and higher IL-10 expression within non-T cells in spleen, and a tendency towards a mucosal increase in TGF-β[+] Foxp3^{+} regulatory T cells. Our data shows that the gluten-containing standard diet modifies the cytokine pattern of both Foxp3^{−} T cells and Foxp3^{+} regulatory T cells towards a more inflammatory cytokine profile. This immune profile may contribute to the higher type 1 diabetes incidence associated with gluten intake.

Keywords: Cytokines; Foxp3 regulatory T cells; Gluten; Mucosal immunology; T cells; Type 1 diabetes.

Introduction

Type 1 diabetes (T1D) and coeliac disease are both diseases of autoimmune origin. In T1D tolerance to beta cell antigens is broken, leading to expansion of autoreactive T cells that attack pancreatic beta cells with subsequent loss of insulin production. In addition to being a disease-initiating factor in coeliac disease, there is evidence that gluten affects the development of T1D. A gluten-free (GF) diet is found to have a protective effect on the development of disease, as observed in the non-obese diabetic (NOD) mice where a GF diet prevents diabetes.[1] Studies have found that antibody production against a wheat-storage globulin (Glb1) was higher in patients with T1D compared with controls[2] and newly diagnosed patients with T1D have enhanced T-cell reactivity to gluten.[3] The highest incidence of diabetes in experimental animal models of T1D is found in animals on a wheat-based diet,[4] and a cereal-based diet promoted T1D development in two animal models of T1D, the NOD mice and the biobreeding rat.[5,6] The diabetogenic potential of gluten seems to be dependent on the time of gluten introduction, both in biobreeding rats[7] and NOD mice, as delayed exposure to dietary gluten in newborn NOD mice was sufficient to reduce later development of T1D.[8] This is consistent with human studies where early introduction of gluten-containing cereals to children increases the risk of developing islet autoantibodies.[9]

Evidence points towards involvement of the intestinal immune system in the development of T1D. In NOD mice the lymphocytes infiltrating the islets express the gut-associated lymphoid tissue-specific integrin α_4/β_7,[10] and mesenteric lymphocytes transfer diabetes from NOD mice to non-diabetic mice, which indicates that diabetogenic T cells are activated in the intestinal compartment before infiltrating the pancreas.[11] Moreover, gastrointestinal changes (increased intestinal permeability and

enteropathy) are found in both human T1D patients[12] and in animal models of T1D.[13]

Few studies have been performed to clarify how dietary gluten affects the immune system. NOD mice on a wheat-based diet have a T helper type 1 (Th1) cytokine bias in the gut,[14] and in biobreeding rats a cereal-based diet induces a Th1 cytokine profile in pancreas-infiltrating cells.[15] Moreover, gluten has been shown to directly activate dendritic cells.[16,17] Hence, evidence points towards a role for gluten in T1D, perhaps through a complex interplay between gluten, predisposing genes and mucosal immunity. A better understanding of the effect of gluten on the mucosal immune system may help to explain the observed link between gluten intake and the development of T1D.

Regulatory T cells (Tregs) play an important role in the control of peripheral potentially autoreactive T cells, that have escaped central deletion in thymus. Autoimmune diseases may develop through an altered balance between self-reactive T cells and Tregs.[18] The Tregs include a number of different T-cell subsets, including the forkhead box p3 transcription factor (Foxp3) expressing cells. Foxp3-expressing Tregs are a constitutively occurring T-cell sub-population whose deficiency is associated with development of autoimmune diseases, and that are involved in the control of the overall adaptive immune response both in health and disease.[19] Foxp3$^+$ Tregs are able to promote bystander suppression and to block an inflammatory response by regulation of various cell types, including CD4$^+$ and CD8$^+$ T cells, B cells and antigen-presenting cells.[20]

Foxp3$^+$ Tregs mediate their suppression through cell contact-dependent mechanisms, by secretion of immuno-suppressive cytokines such as transforming growth factor-β (TGF-β), interleukin-10 (IL-10) and IL-35 or by competing with T effector (Teff) cells for cytokines, e.g. IL-2, that are necessary for survival and expansion of both Tregs and Teff cells.[20] Despite the fact that Foxp3$^+$ Tregs are considered to be a constitutive, regulatory T-cell population, it has become evident that this subset is not always stable. When exposed to appropriate cytokine signals, a small fraction of Foxp3$^+$ CD4$^+$ T cells retains plasticity to differentiate into Teff cells, with the potential to produce inflammatory cytokines such as interferon-γ (IFN-γ) and so lose their regulatory activity.[21,22] Recently, IL-17-producing Foxp3$^+$ cells have been identified in human peripheral blood, tonsils[23] and in small intestinal lamina propria.[24] Interleukin-17 is usually secreted by Th17 cells, a subpopulation of CD4$^+$ T cells, which are instrumental in the response against microbial infection, but Th17 cells are also potent inducers of tissue inflammation and associated with organ-specific autoimmunity.[25] Considerable evidence exist in animals as well as humans for the importance of IFN-γ and IL-17 in the development and progression of autoimmune diseases, and for TGF-β and IL-10 as key mediators in maintaining

T-cell tolerance to self by regulating differentiation and homeostasis of Tregs and Teff cells.[26,27]

The aim of the present study was to clarify the effect of the gluten-containing standard (STD) diet on the cytokine pattern in Foxp3$^-$ and Foxp3$^+$ T cells isolated from mucosal lymphoid tissues: Peyer's patches (PP), mesenteric lymph nodes (MLN) and pancreatic lymph nodes (PLN), and from non-mucosal lymphoid compartments: spleen (S) and inguinal lymph nodes (ILN), and to compare these results with similar experiments using mice receiving the diabetes-protective GF diet. The experiments were performed using fully immunocompetent BALB/c mice. Gluten-induced changes in the cytokine pattern of T cells may have an important impact on the development of autoimmune disease in susceptive individuals because the overall effect of an inflammatory response is determined by the balance between anti-inflammatory and pro-inflammatory cytokines.

Materials and methods

Animals/diets

Timed pregnant BALB/cA BomTac mice were purchased from Taconic Europe A/S, Ejby, Denmark. Mice were kept in specific pathogen-free animal facilities with free access to water and food, the temperature was 22 ± 2° and light was switched off from 18.00 to 06.00, with air changed 16 times per hour, and humidity maintained at 55 ± 10%. Mice with first-generation female offspring were divided into two groups 7 days after delivery. One group of mice received the gluten-containing STD altromin diet and the other group of mice received the GF modified altromin diet (Altromin, Lage, Germany), both diets have previously been used at the Bartholin Institute to study the effect of a GF diet on diabetes incidence in NOD mice.[1,28] First-generation female offspring ($n = 6$), in each dietary group, were used in the study when 6 weeks old. Both experimental diets were nutritionally adequate with a similar level of protein, amino acids, minerals, vitamins and trace elements, only the protein source differed between the two diets. The weight of the mice was followed and growth curves were comparable between the two groups of mice. The exact composition of the STD and the GF diets is given in earlier papers.[1,28] The animal experiments were carried out with approval from The National Animal Experimental Board, and experiments were performed in accordance with international guidelines for the care and use of laboratory animals.

Surface and intracellular cytokine staining and flow cytometry

Single-cell suspensions were prepared from S, ILN, MLN, PP and PLN from mice in each diet group. Cells from

each organ were pooled because of the limited number of cells obtained from PLN. Cell suspensions were separated into two groups, one for stimulation with ionomycin (2 μg/ml) and PMA (50 ng/ml) for 4 hr in the presence of Golgi-stop, the rest of the cells remained unstimulated as controls. The cells were kept in a tissue culture incubator at 37°. Then cells were pre-incubated with Fc block CD16/CD32 (2.4G2, IgG2b; BD Biosciences, Franklin Lakes, NJ) and stained with peridinin chlorophyll protein-conjugated rat anti-mouse CD3 monoclonal antibody (17A2; IgG2b; BD Biosciences). Cell pellets were resuspended with fixation/permeabilization solution (Mouse regulatory T cell Staining kit, cat. no 88–8111; eBioscience, San Diego, CA) following the manufacturer's protocol. Fc block CD16/CD32 was added and the labelled antibodies were added to the cells, and stained for 30 min at 4°. We used phycoerythrin-conjugated rat anti-mouse Foxp3 monoclonal antibody (FJK-16s, IgG2a; eBioscience), Alexa Fluor 488-conjugated rat anti-mouse IL-2 (JES6-5H4, IgG2b; BD Biosciences), Alexa Fluor 488-conjugated rat anti-mouse IL-4 (11B11, IgG1; BD Biosciences), FITC-conjugated rat anti-mouse IL-10 (JES5-16E3, IgG2b), Alexa Fluor 488-conjugated rat anti-mouse IFN-γ (XMG1.2; IgG1κ; BD Biosciences), biotin-conjugated rat anti-mouse TGF-β (A75-3; IgG2a, κ; BD Biosciences), streptavidin (cat.no 349024; BD Biosciences) and Alexa Fluor 488-conjugated rat anti-mouse IL-17 (17B7; IgG2a,κ; eBioscience). Isotype control antibodies were used to determine the amount of non-specific binding, and propidium iodide was used to exclude dead cells. Cells were analysed by flow cytometry using a FACSscan (BD Biosciences), and data were analysed using CELLQUEST software (BD Biosciences). Cytokine expression analysis was performed by gating according to the CD3 and Foxp3 parameters. The results of the study are from the PMA/ionomycin-stimulated cells because no, or very few, cytokine-positive cells were found in unstimulated controls.

Data analysis

Statistical analyses were performed using NCSS 2004/GESS STATISTICAL software, Kaysville, UT. Differences between the diets were determined using a one-sample t-test. We tested diet (STD versus GF) -induced differences between non-mucosal (S, ILN) and mucosal (MLN, PP, PLN) lymphoid compartments to determine whether dietary gluten affects the balance of pro-inflammatory versus anti-inflammatory cytokines in Foxp3$^+$ Tregs as well as Foxp3$^-$ T cells at systemic lymphoid organs or only locally; at mucosal lymphoid organs. Based on the concept of the common mucosal immune system[29] we investigated various mucosal lymphoid organs; MLN, PP, PLN. Differences at $P < 0.05$ were considered statistically significant. Only data showing substantial diet-induced

differences are shown in the Results section. Graphic presentation of the results was performed using the SIGMA PLOT 9.0 (Systat Software, San Jose, CA) and GRAPHPAD PRISM version 5 software (Graphpad Software, La Jolla, CA).

Results

The proportion of IL-2$^+$ T cells was increased in mice receiving the STD diet

The IL-2 expression within Foxp3$^+$ T cells was significantly higher ($P < 0.05$) in mucosal lymphoid tissues (MLN, PP, PLN) in mice receiving the STD compared with the GF diet (Fig. 1). The proportion of IL-2$^+$ Foxp3$^+$ T cells was on average 21% higher in mucosal lymphatic tissue in mice receiving the STD diet than in mice on the GF diet. No diet-induced difference was found in non-mucosal lymphoid compartments (S, ILN). When evaluating the proportion of IL-2$^+$ Foxp3$^-$ T cells, we found an average of 17% more IL-2$^+$ Foxp3$^-$ T cells in mice on the STD diet, both in non-mucosal (S, ILN) and mucosal (MLN, PP, PLN) lymphoid compartments ($P < 0.01$).

The STD diet increases the proportion of IL-4$^+$ Foxp3$^+$ T cells both in non-mucosal lymphoid compartments and in mucosal lymphoid tissues

We found an overall (S, ILN, MLN, PP, PLN) increase ($P < 0.01$) in IL-4$^+$ Foxp3$^+$ T cells, in mice on the STD diet compared with mice on the GF diet (Fig. 2). The proportion of IL-4$^+$ Foxp3$^+$ T cells was on average 26% higher in mice receiving the STD compared with mice on the GF diet. No substantial differences between the diets were found in IL-4$^+$ Foxp3$^-$ T cells (data not shown).

Increased proportion of IL-10$^+$ non-T cells in spleens from mice on the GF diet

Intracellular staining for IL-10$^+$ Foxp3$^+$ T cells revealed that these cells were present both in non-mucosal and mucosal-associated lymphoid tissue, varying from 5·7% in S to 16·8% in PP (gated on 100% Foxp3$^+$ CD3$^+$) (Fig. 3). Diet-induced differences in this cell population were not found. Moving the gate to non-T cells (CD3$^-$ cells) we found no significant differences in IL-10$^+$ CD3$^-$ cells, according to diet, when comparing any difference between the mucosal lymphoid tissues (MLN, PP, PLN) and the non-mucosal lymphoid compartments (S, ILN). Only a marked difference between the two diets in IL-10$^+$ CD3$^-$ cells was observed in S. Mice on the STD diet were found to have a low proportion (0·7%) of IL-10$^+$ CD3$^-$ cells, whereas mice on the GF diet had a
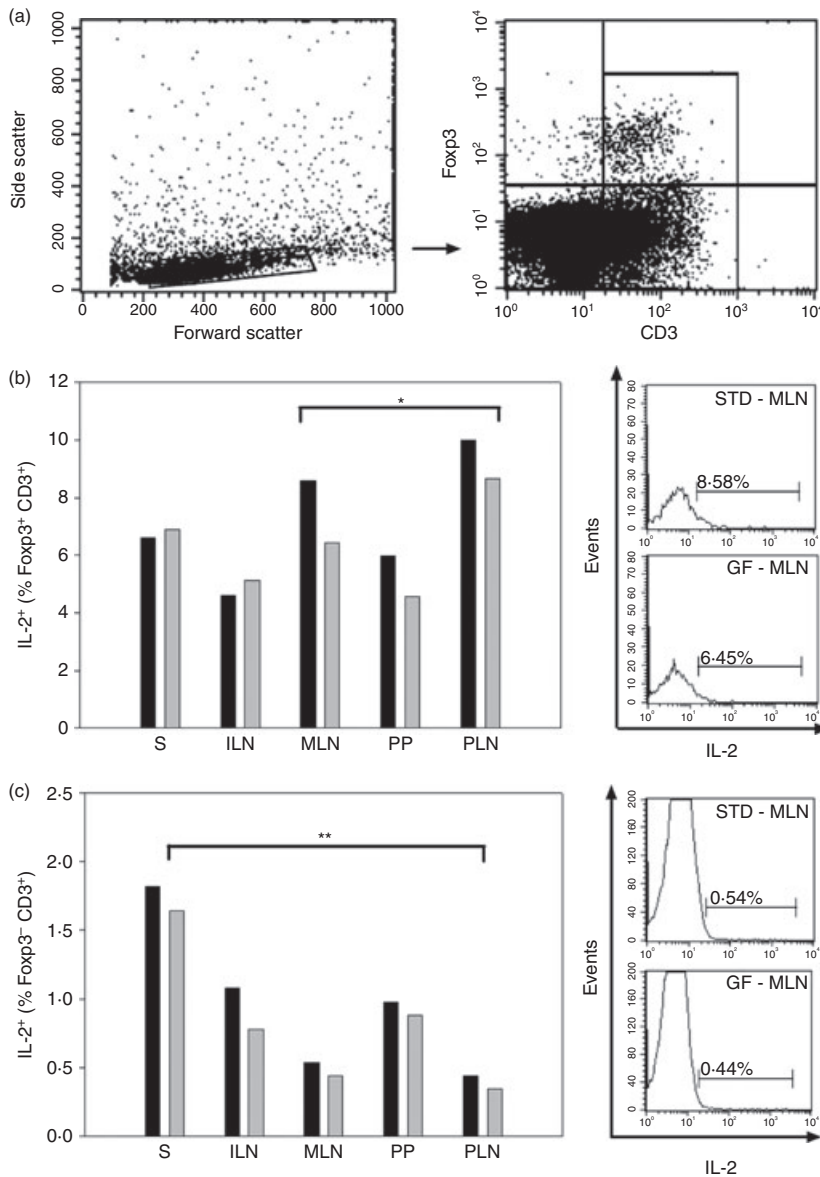
Figure 1. Intracellular staining and FACS analysis of interleukin-2 (IL-2)$^+$ T cells. (a) Representative plot of lymphocyte and the subsequent CD3$^+$ gate. (b) The bars represent the proportion of IL-2$^+$ cells (gated on 100% Foxp3$^+$CD3$^+$) in mice receiving the standard (STD) diet compared with the gluten-free (GF) diet. We found significant ($P < 0.05$) differences between the diets in mucosal lymphoid tissues. Histogram from mesenteric lymph nodes (MLN), showing the proportion of IL-2$^+$ Foxp3$^+$ T cells in STD mice compared with GF mice. (c) Significant ($P < 0.01$) diet-induced changes in the proportion of IL-2$^+$ Foxp3$^-$ T cells were found, in all tested lymphoid compartments. Spleen (S); inguinal lymph nodes (ILN); MLN; Peyer's patches (PP) and pancreatic lymph nodes (PLN). Histograms from MLN showing differences in IL-2$^+$ Foxp3$^-$ T cells according to diet. Pools of lymphocytes from six mice in each group were used. Black bars: STD diet. Grey bars: GF diet. *$P < 0.05$. **$P < 0.01$.

> 100% increase in these cells (1·43%). We found no diet induced differences in IL-10$^+$ Foxp3$^-$ T cells (data not shown).

Higher proportion of TGF-β^+ T cells in mice on the GF diet

The proportion of TGF-β^+ Foxp3$^+$ T cells was between 3·75%, in ILN in mice on the STD diet and 8·07% in PLN in the GF mice (Fig. 4). Diet-induced differences were not observed in either S, ILN or MLN. However, mice on the GF diet had a tendency towards a higher proportion of TGF-β^+ Foxp3$^+$ T cells in PP (5·2% in STD versus 8·1% in GF) and in PLN (4·0% in STD versus 8·1% in GF). Interestingly, we found a consistently higher proportion ($P < 0.001$) of TGF-β^+ Foxp3$^-$

T cells in mice receiving the GF diet. The GF mice had more than twice as many TGF-β^+ Foxp3$^-$ T cells in all isolated lymphoid organs (S, ILN, MLN, PP, PLN). The highest difference between the diets was found in PP (0·3% versus 0·7%) in mice fed the STD and GF diets.

Marked increase in IFN-γ^+ cells in mice on the STD diet

The proportion of IFN-γ^+ Foxp3$^+$ T cells was increased ($P < 0.01$) in mice receiving the STD diet compared with the GF diet, in all isolated lymphoid organs (S, ILN, MLN, PP, PLN) (Fig. 5). The diet-induced differences were highest in MLN and PLN. In PLN from mice on the STD we found 12·18% cells being IFN-γ^+ compared with
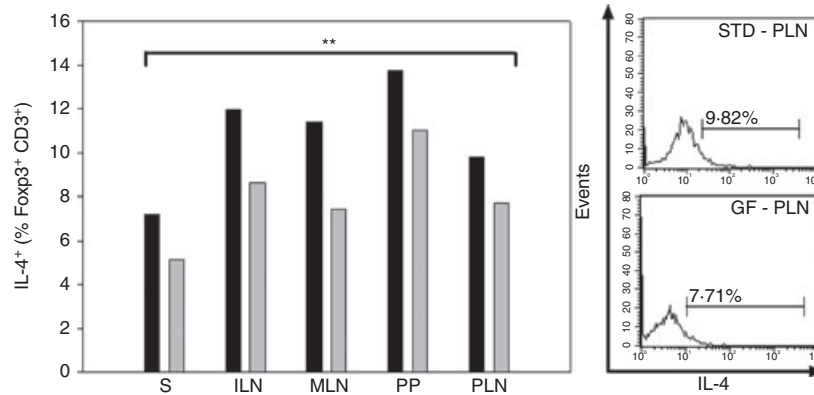
Figure 2. Intracellular staining and FACS analysis of interleukin-4 (IL-4)$^+$ T cells. The bars represent the proportion of IL-4$^+$ cells (gated on 100% Foxp3$^+$ CD3$^+$), in mice receiving the standard (STD) diet versus the gluten-free (GF) diet. We found a significantly ($P < 0.01$) higher proportion of IL-4$^+$ Foxp3$^+$ T cells in STD mice in all isolated lymphoid tissues. Histogram from pancreatic lymph nodes (PLN), is shown. The indicated values represent the percentage of IL-4$^+$ Foxp3$^+$ T cells. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and PLN. Pools of lymphocytes from six mice in each group were used. Black bars: STD diet. Grey bars: GF diet. **$P < 0.01$.

6.98% IFN-γ^+ cells in the PLN isolated from GF mice, when gated on 100% Foxp3$^+$ CD3$^+$. In Foxp3$^-$ T cells, we also found an increase in IFN-γ^+ cells in STD mice ($P < 0.01$). The average proportion of IFN-γ^+ Foxp3$^-$ T cells in all lymphoid organs was 36% higher in STD mice than in the GF mice. Furthermore, we investigated differences in IFN-γ expression within non-T cells. The MLN and PLN had a tendency towards a higher proportion of IFN-γ^+ non-T cells, although the higher proportion was not significant when calculating on an overall mucosal (MLN, PP, PLN) difference. In MLN, mice on the STD diet had 2.35% IFNγ^+ CD3$^-$ cells compared with 1.7% in mice receiving the GF diet. The same pattern was found in PLN with 2.67% IFN-γ^+ CD3$^-$ cells in STD mice and 1.1% in GF mice.

Increased proportion of IL-17$^+$ T cells in mice on the STD diet

The gluten-containing STD diet significantly increased ($P < 0.05$) the proportion of IL-17$^+$ CD3$^+$ cells in both non-mucosal and mucosal lymphoid compartments (S, ILN, MLN, PP, PLN) (Fig. 6). The overall increase of mean was 60% between the diets, with the highest diet-induced difference in PP and PLN. Surprisingly, we found IL-17$^+$ Foxp3$^+$ T cells in all organs. The lowest proportion was found in PP in mice on the GF diet, with 2.3% IL-17$^+$ Foxp3$^+$ T cells and the highest proportion in MLN in mice receiving the STD diet with 7.9% IL-17$^+$ Foxp3$^+$ T cells (gated on 100% Foxp3$^+$ CD3$^+$ cells). Gluten induced a significant ($P < 0.05$) increase in IL-17$^+$ Foxp3$^+$ T cells in mucosal lymphoid tissues (MLN, PP, PLN), but no significant difference between the diets was found in non-mucosal lymphoid compartments (S, ILN). The proportion of IL-17$^+$ Foxp3$^+$ T cells was on average 39% higher in mucosal lymphoid tissue

in mice receiving the STD diet than in mice on the GF diet. When analysing the effect of the two diets we found no substantial diet-induced differences in IL-17$^+$ Foxp3$^-$ T cells (data not shown).

Dietary gluten alters the balance of pro-inflammatory and anti-inflammatory cytokines in T cells

The gluten-containing STD diet induces a pro-inflammatory (IL-17, IFN-γ) cytokine pattern in Foxp3$^-$ T cells in all lymphoid tissues tested, and a decreased level of anti-inflammatory cytokines (IL-10, TGF-β) in ILN, MLN, PP, PLN (Fig. 7). Furthermore, dietary gluten induces a pro-inflammatory (IL-17, IFN-γ) cytokine pattern in Foxp3$^+$ T cells and an increased level of IL-2 and IL-4 in all lymphoid tissues tested, and a decreased level in anti-inflammatory cytokines (IL-10, TGF-β) in S, PP and PLN.

Discussion

The present study shows that gluten intake changes the cytokine pattern in Foxp3$^+$ and Foxp3$^-$ T cells towards a pro-inflammatory cytokine profile. The protective role of a GF diet[1] is not associated with changes in the number of the Foxp3$^+$ Tregs[30] but could be the result of an increase in the expression of immunosuppressive cytokines such as TGF-β and, to a lesser extent, IL-10. Conversely, the STD, gluten-containing diet increased the level of potent pro-inflammatory (IL-17, IFN-γ) cytokines, as well as of IL-2 and IL-4.

The exact mechanism by which dietary gluten influences cytokine profiles in Foxp3$^+$ and Foxp3$^-$ T cells is not known. We have previously documented that the diabetes-preventive GF diet decreases the number of caecal bacteria and is associated with quantitative changes in the composition of the bacterial flora in NOD mice.[31]
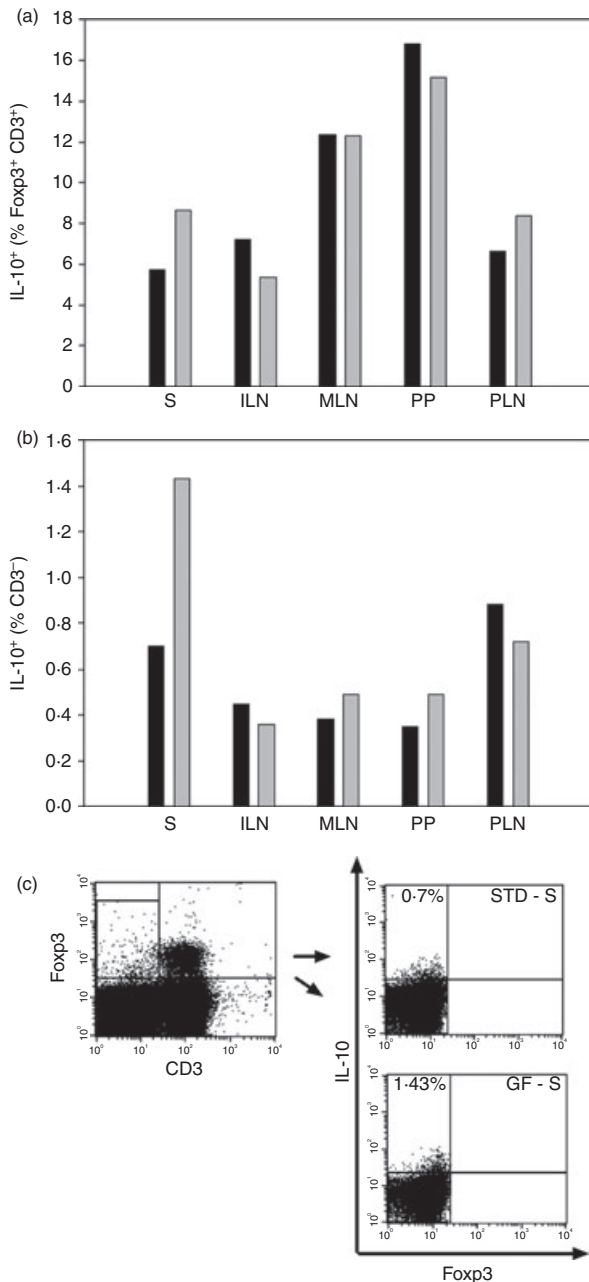
Figure 3. Intracellular staining and FACS analysis of interleukin-10 (IL-10)$^+$ cells. (a) The proportion of IL-10$^+$ Foxp3$^+$ T cells in mice on the standard (STD) versus the gluten-free (GF) diet. No significant diet-induced changes were found in this cell population. (b) When gating on 100% CD3$^-$ cells, we found no significant diet-induced changes when comparing non-mucosal lymphoid tissues with mucosal-associated lymphoid tissues. Although a higher proportion of IL-10$^+$ non-T cells was observed in spleen (S). (c) Dot plots showing the CD3$^-$ gate and the proportion of IL-10$^+$ CD3$^-$ cells isolated from S in mice receiving the STD diet versus the GF diet. Spleen; inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and pancreatic lymph nodes (PLN). Pools of lymphocytes from six mice in each group were used. Black bars: STD diet. Grey bars: GF diet.

Whether the gluten-induced changes in cytokine profiles are mediated directly or via intestinal microflora have not yet been clarified by gnotobiotic germ-free experiments.

In this study, based on the concept of the common mucosal immune system[29] we clarify how dietary gluten influences cytokine pattern of Foxp3$^+$ and Foxp3$^-$ T cells in mucosal and non-mucosal lymphoid compartments. PLNs, the draining lymph nodes of pancreas, are grouped into the mucosal compartment, because mucosa-associated, β_7-integrin^{high} lymphocytes accumulate in early phases of islet inflammation in the pancreas of NOD mice.[10] The mucosal character of PLNs is also supported by recent findings[32] documenting that B cells in pancreatic lymph nodes of NOD mice express integrin α_4/β_7 and that the integrin $\alpha_4\beta_7$/MAdCAM-1 mucosal adhesion pathway is important for their migration to PLN.

Our data provide new insight into the effect of dietary gluten on immune reactivity and have implications for understanding the observed dietary modification of T1D development in NOD mice.[1] Because NOD mice are lymphophenic and display other immune dysbalances, we studied the effect of dietary gluten in fully immunocompetent BALB/c mice. Hence, we observed which immunological changes gluten exposure induces in a healthy animal model at 6 weeks. The age of the animals was chosen to reflect a 'pre-diabetic time-point' to allow later comparison with pre-diabetic NOD mice. Our results shed more light on immune status associated with exposure to dietary gluten, that has been reported as an environmental factor in development of T1D, both in animal models[7,8,15] and in humans.[9] The results are important because they illustrate how a dietary protein directly changes the balance of pro-inflammatory and anti-inflammatory cytokines in T cells.

Multiple studies, both in humans and in mice, propose a deficiency in either the function or the frequency of Tregs,[33,34] as an explanation of development of autoimmunity. Hence it has been tested whether a reduced number of CD4$^+$ CD25$^+$ T cells are found in patients with T1D compared with controls. The first human study indicated that this was the case,[35] but subsequent studies failed to repeat these findings.[36,37] CD4$^+$ CD25$^+$ T cells from T1D patients have a reduced capacity to suppress proliferation of co-cultured CD4$^+$ CD25$^-$ T cells, compared with controls.[38,39] This suggests a defective suppressor function in CD4$^+$ CD25$^+$ T cells in patients with T1D and could be a result of changes in the cytokine pattern of the Tregs, as CD4$^+$ CD25$^+$/CD4$^+$ CD25$^-$ co-cultures from T1D patients produced more IFN-γ, and less IL-10 than healthy controls.[38] These findings are similar to our present findings in animals receiving the STD diet, supporting the notion that functional changes in Tregs could be involved in the susceptibility for the development of T1D.
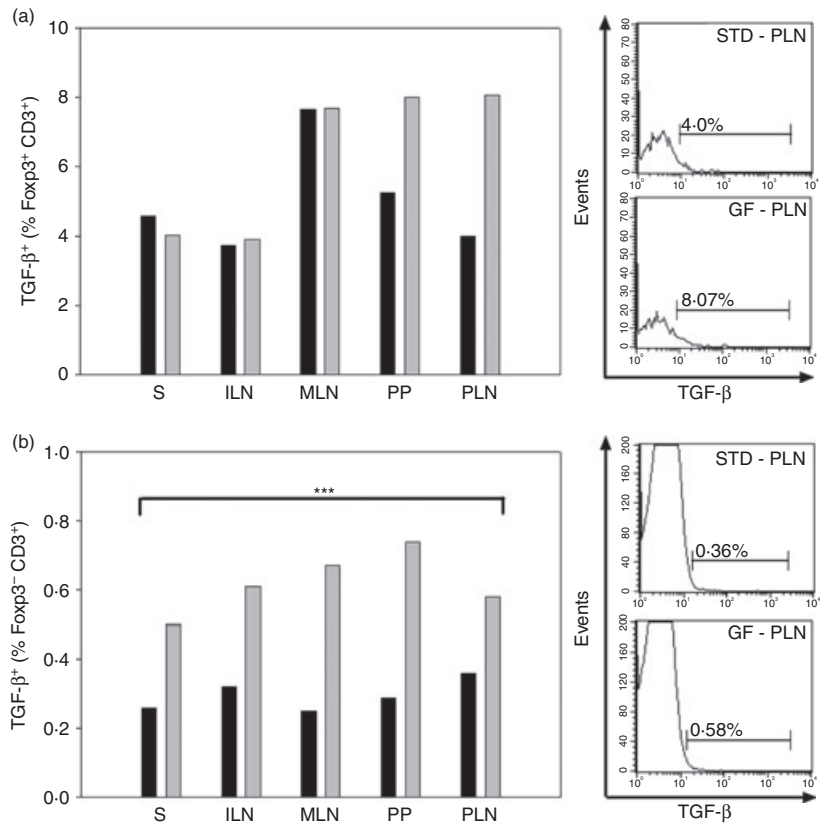
Figure 4. Intracellular staining and FACS analysis of transforming growth factor-β (TGF-β)$^+$ T cells. (a) Bars show the proportion of TGF-β^+ Foxp3$^+$ T cells. Mice on the gluten-free (GF) diet have a tendency towards a higher proportion of TGF-β^+ Foxp3$^+$ T cells in Peyer's patches (PP) and pancreatic lymph nodes (PLN). Histograms show the diet-induced differences in PLN. (b) The distribution of TGF-β^+ Foxp3$^-$ T cells. A significantly ($P < 0.001$) higher proportion was found in all lymphoid organs isolated from mice on the GF diet. Histogram showing diet-induced differences in PLN. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); PP and PLN. Pools of lymphocytes from six mice in each group were used. Black bars: STD diet. Grey bars: GF diet. ***$P < 0.001$.

The regulatory cytokines IL-10 and TGF-β are of major importance for mediating the suppressive activity of Tregs.[40] The importance of IL-10 is shown in studies where the dampening of autoaggressive CD8 cells is associated with production of IL-4 and IL-10 by Tregs.[41] This is contrary to studies showing that the regulatory function of IL-10-secreting islet-specific Tregs clones, seems to be cell–cell contact-dependent and independent of IL-10, as blocking of IL-10 or TGF-β had no effect on the ability of these cells to work as suppressors.[42] Other studies suggest an important suppressive role for TGF-β, based on the association between a reduction in suppression by Tregs with blockade of TGF-β[43,44] and the fact that TGF-β has a broad inhibitory effect on the entire immune system. We observed that the GF diet significantly increases the level of TGF-β in Foxp3$^-$ T cells, as well as a tendency towards a mucosal increase of TGF-β in Foxp3$^+$ T cells. Moreover, Foxp3$^+$ T cells in all lymphoid organs produced IL-10, although we found no diet-induced differences in these cells. The proportion of IL-10$^+$ Foxp3$^+$ T cells was highest in MLN and PP, as previously described.[27] The GF diet increased the proportion of splenic IL-10$^+$ non-T cells.

In our recent study we have documented that the gluten-containing STD diet does not change the proportion of Foxp3$^+$ T cells in either mucosal or non-mucosal lymphoid organs of BALB/c mice.[30] In this present study we documented cytokine changes, which could reflect diet-induced functional changes within the Foxp3$^+$ T cells. These findings may suggest that impaired regulatory function of Foxp3$^+$ Tregs rather than their absolute number is of importance in progression to autoimmunity (T1D). The importance of gluten exposure combined with a defect in Foxp3$^+$ T cells is in line with recently published data showing that sensitization to gliadin and partial systemic depletion of Foxp3$^+$ T cells induces insulitis in the non-obese diabetic-DQ8 mice, that do not spontaneously develop diabetes.[45]

During the last years, the Th1/Th2 paradigm has expanded, following the discovery of a third subset of Th cells that produce IL-17 (Th17).[46] They differentiate in response to TGF-β and IL-6, and as potent inducers of tissue inflammation they are associated with the pathogenesis of various human inflammatory conditions and autoimmune diseases,[25] including T1D.[47] The Th17 cells seem more resistant to regulatory control than Th1 and Th2 cells.[48] In this study we found an overall higher proportion of IL-17$^+$ CD3$^+$ cells in mice receiving the gluten-containing STD diet, with a surprising increase in mucosal-associated lymphoid tissue (PP and PLN), where the IL-17$^+$ CD3$^+$ cell population more than doubles. These data are in accordance with our recent study documenting a significantly increased proportion of CD4$^+$ IL-17$^+$ (Th17) cells in the PLN of BALB/c mice fed an STD diet.[30]

The detection of IL-17$^+$ Foxp3$^+$ T cells is in line with recently published data. It has been reported that human
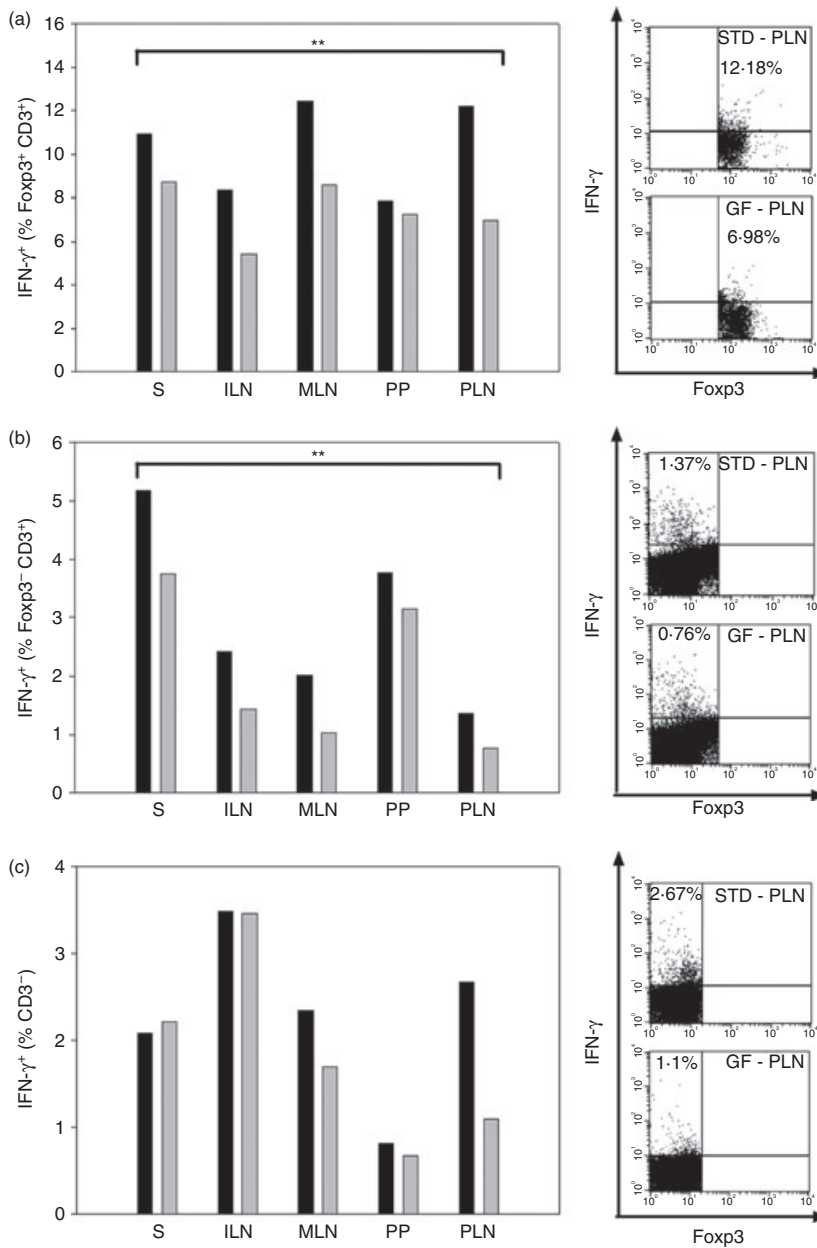
Figure 5. Intracellular staining and FACS analysis of interferon-γ (IFN-γ)$^+$ T cells. (a) Bars show the proportion of IFN-γ$^+$ Foxp3$^+$ T cells with a significant ($P < 0.01$) increase in mice on the standard (STD) diet compared with the gluten-free (GF) diet in all isolated lymphoid tissues. Dot plot of isolated cells from pancreatic lymph nodes (PLN). (b) Bars show a diet-induced increase ($P < 0.01$) in IFN-γ$^+$ Foxp3$^-$ T cells in all tested lymphoid organs. Dot plots of IFN-γ$^+$ Foxp3$^-$ T cells isolated from PLN. (c) Bars represent the proportion of IFN-γ$^+$ non-T cells. Dot plots of IFN-γ$^+$ non-T cells in PLN. Spleen (S); inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and PLN. Pools of lymphocytes from six mice in each group were used. Black bars: STD diet. Grey bars: GF diet. **$P < 0.01$.

peripheral blood and tonsil contain Foxp3$^+$ Tregs with the capacity to produce IL-17 upon activation. These cells are possibly generated at mucosal sites during an inflammatory process.[23] Furthermore, the presence of Foxp3$^+$ RORγ$^+$ (a transcription factor involved in the development of Th17 cells) T cells with the ability to produce IL-17 in the small intestinal lamina propria has been described.[24] We found IL-17$^+$ Foxp3$^+$ T cells in proportions ranging from 2·3% cells in PP to 7·9% in MLN. Interestingly, we found that the proportion of IL-17$^+$ Foxp3$^+$ T cells was affected by gluten intake, as the STD diet increased the proportion of IL-17$^+$ Foxp3$^+$ T cells in mucosal lymphoid tissues (MLN, PP, PLN). Hence, gluten intake induces a functional change in

Foxp3$^+$ Tregs, towards production of highly pro-inflammatory IL-17.

Some Foxp3$^+$ T cells analysed were positive for pro-inflammatory cytokines, which supports recent evidence suggesting considerable plasticity of Foxp3$^+$ Tregs with respect to their capacity to produce cytokines, such as IL-17[24] and IFN-γ.[49] This is consistent with the notion that Foxp3 may be expressed more widely and transiently than initially conceived.[24,50] Contributing to the diverse picture is the fact that human, activated T cells can express Foxp3 although lacking regulatory function,[51,52] and Foxp3 induction *ex vivo* does not confer a regulatory phenotype.[53] Moreover, several recent studies suggest that there is a loss of Foxp3 expression during inflammatory
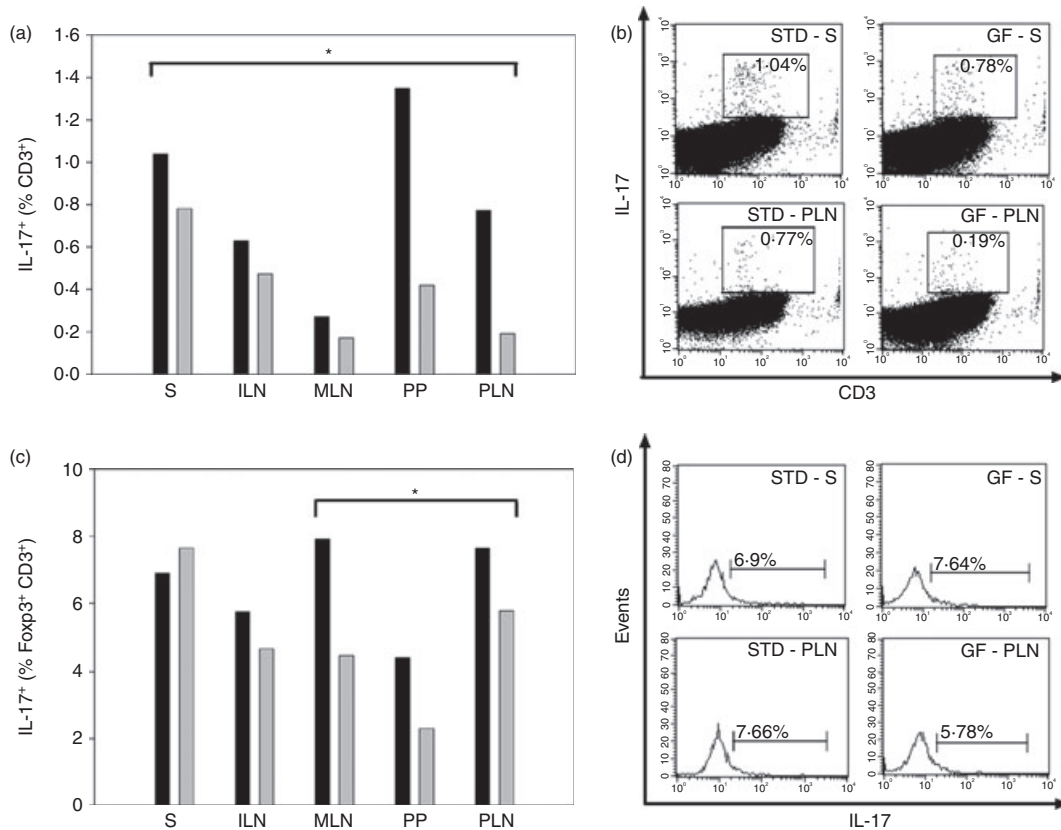
Figure 6. Intracellular staining and FACS analysis of interleukin-17 (IL-17)[+] T cells. (a) Increased ($P < 0.05$) proportion of IL-17^{+} T cells in mice receiving the standard (STD) diet compared with the gluten-free (GF) diet in all tested lymphoid tissues. (b) Dot plots of isolated IL-17^{+} T cells from spleen (S) and pancreatic lymph nodes (PLN). (c) The proportion of IL-17^{+} Foxp3^{+} T cells. With a mucosal increase ($P < 0.05$) in IL-17^{+} Foxp3^{+} T cells in mice receiving the STD diet. (d) Histograms from S and PLN showing the proportion of IL-17^{+} Foxp3^{+} T cells. Spleen; inguinal lymph nodes (ILN); mesenteric lymph nodes (MLN); Peyer's patches (PP) and PLN. Pools of lymphocytes from six mice in each group were used. Black bars: STD diet. Grey bars: GF diet. *$P < 0.05$.

conditions, and that low Foxp3 expression predisposes for autoimmune diseases in mice susceptible for disease.[54] Therefore it seems that not all Foxp3^{+} T cells possess a regulatory function, either because of a lower amount, or through unstable Foxp3 expression. It is suggested that some Tregs, under specific inflammatory conditions, become 'effector-like' T cells, likely to play an important role in autoimmune diseases.[22] This explanation could apply for our findings of gluten-induced pro-inflammatory cytokines in Foxp3^{+} T cells, changes that could affect the ability of Tregs to suppress autologous effector T cells.

The site of Tregs suppression *in vivo* is not limited to lymphoid organs, and the ability of Tregs to migrate and exert their function in inflamed tissue is important for their function *in vivo*.[55] Studies in murine models of T1D indicate that Tregs exert their function within the target organ undergoing autoimmune attack, and also in the associated, draining lymph nodes, so it could be relevant to test whether the observed differences in T cells are also

present in pancreatic tissue and in intestinal lamina propria. Furthermore, it is important to notice that the molecular mechanism of suppression used by Tregs seems to differ depending on being present in either lymphoid tissue or non-lymphoid tissue.[20]

In summary, our study shows that the STD, gluten-containing diet changes the cytokine pattern in both Foxp3^{−} and Foxp3^{+} T cells towards a more pro-inflammatory cytokine profile with higher levels of IL-17, IFN-γ, IL-2 and IL-4. Inversely, a diabetes-protective, gluten-free diet induces an anti-inflammatory cytokine profile with higher IL-10 production by non-T cells in S and a higher proportion of Foxp3^{−} T cells (and a tendency of mucosal increase in Foxp3^{+} T cells) secreting TGF-β.

We suggest that a failure in the development of a proper regulatory immune response to environmental stimuli may contribute to development of T1D in genetically predisposed individuals. Certain environmental stimuli, such as gluten, induce inflammatory changes in T cells that negatively influence mucosal immune
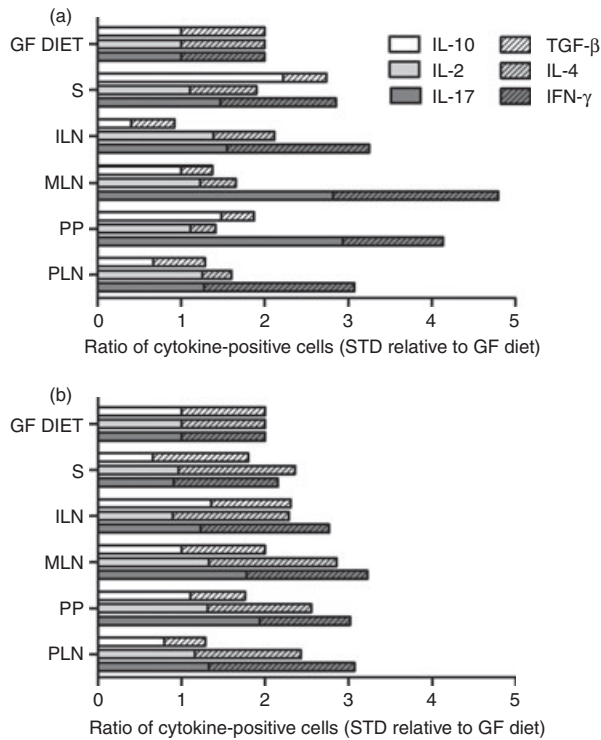
Figure 7. The overall gluten-induced changes in the cytokine pattern in Foxp3⁻ and Foxp3⁺ T cells. (a) Foxp3⁻ T cells. Ratio of cytokine positive Foxp3⁻ T cells, from mice on the standard (STD) diet relative to the gluten-free (GF) diet. Dietary gluten induces a pro-inflammatory [interleukin-17 (IL-17), interferon-γ (IFN-γ)] cytokine pattern in Foxp3⁻ T cells in all lymphoid tissues tested, and a deceased level of anti-inflammatory [IL-10, transforming growth factor-β (TGF-β)] in inguinal lymph node (ILN), mesenteric lymph node (MLN), Peyer's patches (PP), and pancreatic lymph node (PLN). (b) Foxp3⁺ T cells. Ratio of cytokine-positive Foxp3⁺ T cells, from mice on the STD diet relative to the GF diet. Dietary gluten induces a pro-inflammatory (IL-17, IFN-γ) cytokine pattern in Foxp3⁺ T cells and an increased level of IL-2 and IL-4 in all lymphoid tissues tested, and a decreased level in anti-inflammatory (IL-10, TGF-β) in spleen (S), Peyer's patch (PP) and pancreatic lymph nodes (PLN). Inguinal lymph nodes (ILN).

homeostasis, which could affect the diabetogenic process in the pancreas towards development of autoimmunity.

Acknowledgements

Disclosure

The authors declare no conflicts of interest.

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3.2.2. Publication VI.

Palová-Jelínková L, Dáňová K, Drašarová H, Dvořák M, Funda DP, **Fundová P,** Kotrbová-Kozak A, Cerná M, Kamanová J, Martin SF, Freudenberg M, Tučková L. Pepsin Digest of Wheat Gliadin Fraction Increases Production of IL-1β via TLR4/MyD88/TRIF/MAPK/NF-κB Signaling Pathway and an NLRP3 Inflammasome Activation. *PLoS One,* **2013; 8(4):e62426**. IF (2015) = 3.057

Gluten fraction of wheat is the known triggering substance in celiac disease, a complex inflammatory disorder, in which a lot of studies dealt with the adaptive immune mechanisms. This is partly due to the fact, that both the triggering agent and the culprit HLA molecules are well known (Sollid, 2002). Deamidation of gluten peptides by tissue transglutaminase increases their binding affinity to the HLA-DQ2 or HLA-DQ8 on APCs, thus increasing their chances to prime gluten-reactive T cells (Jabri and Sollid, 2009). However, significantly less attention has been given to innate immune mechanisms utilized by gluten and/or wheat proteins. Apart from the activated HLA-DQ2 or HLA-DQ8 restricted gliadin-specific T cells various cytokines such as IL-1β, IL-6, IL-15, IL-23 are produced by innate immune cells contribute to the pathology of the disease (Jabri and Sollid, 2006). It has also been shown, including in our lab, that wheat gluten or gliadin fragments cause functional maturation of dendritic cells (Nikulina et al., 2004; Palova-Jelinkova et al., 2005).

In this study we investigated the innate signaling pathways of pepsin digest of wheat gliadin fraction (PDWGF). Using PBMCs and monocytes from active celiac disease patients and healthy controls, we documented significantly increased secretion of IL-1β and IL-1α and slightly increased production of IL-18 in response to PDWGF in CD patients. We then showed that PDWGF leads to de novo pro IL-1β synthesis and that the follow up processing is caspase-1 dependent. IL-1β secretion was promoted by K^+ efflux and independent of P2X7. Using set of MAPK inhibitors we showed that the pro-IL-1β synthesis is induced via the MAPK-NF-κB pathway. Experiments on KO mice then documented that the caspase-1 dependent induction of IL-1β also requires ASC and NLRP3. Following the documentation of NF-κB pathway in IL- β we then explored the upstream TLR signaling pathway using TLR2, 4, 2/4 as well as MyD88 and TRIF KO mice and found the IL-1β secretion is dependent on TLR4, MyD88, and TRIF and influenced by TLR2.

In conclusion, we documented that pepsin digest of wheat gliadin activates innate immune signaling via TLR2/4/MyD88/TRIF/MAPK/NF-κB and the NLRP3 inflammasome pathways. This study on the role of innate immune mechanisms of gliadin actions is also relevant to type 1 diabetes (Funda et al., 2008; Visser et al., 2009), in which more likely its innate than adaptive immune mechanisms influence the development (or prevention - see next paper) of type 1 diabetes.

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PLOS ONE

Pepsin Digest of Wheat Gliadin Fraction Increases Production of IL-1β via TLR4/MyD88/TRIF/MAPK/NF-κB Signaling Pathway and an NLRP3 Inflammasome Activation

Lenka Palová-Jelínková[1]*, Klára Dáňová[1], Hana Drašarová[1], Miloš Dvořák[2], David P. Funda[1], Petra Fundová[1], Anna Kotrbová-Kozak[3], Marie Černá[3], Jana Kamanová[1], Stefan F. Martin[4], Marina Freudenberg[5], Ludmila Tučková[1]

1 Institute of Microbiology, Department of Immunology, Academy of Sciences of the Czech Republic, Prague, Czech Republic, 2 1st Medical Faculty, Charles University, Prague, Czech Republic, 3 Third Faculty of Medicine, Charles University, Prague, Czech Republic, 4 Allergy Research Group, Department of Dermatology, University Medical Center Freiburg, Freiburg, Germany, 5 Max Planck Institute for Immunobiology and Epigenetics, Freiburg, Germany

Abstract

Celiac disease (CD) is a gluten-responsive, chronic inflammatory enteropathy. IL-1 cytokine family members IL-1β and IL-18 have been associated with the inflammatory conditions in CD patients. However, the mechanisms of IL-1 molecule activation in CD have not yet been elucidated. We show in this study that peripheral blood mononuclear cells (PBMC) and monocytes from celiac patients responded to pepsin digest of wheat gliadin fraction (PDWGF) by a robust secretion of IL-1β and IL-1α and a slightly elevated production of IL-18. The analysis of the upstream mechanisms underlying PDWGF-induced IL-1β production in celiac PBMC show that PDWGF-induced de novo pro-IL-1β synthesis, followed by a caspase-1 dependent processing and the secretion of mature IL-1β. This was promoted by K+ efflux and oxidative stress, and was independent of P2X7 receptor signaling. The PDWGF-induced IL-1β release was dependent on Nod-like receptor family containing pyrin domain 3 (NLRP3) and apoptosis-associated speck like protein (ASC) as shown by stimulation of bone marrow derived dendritic cells (BMDC) from NLRP3$^{-/-}$ and ASC$^{-/-}$ knockout mice. Moreover, treatment of human PBMC as well as MyD88$^{-/-}$ and Toll-interleukin-1 receptor domain-containing adaptor-inducing interferon-β (TRIF)$^{-/-}$ BMDC illustrated that prior to the activation of caspase-1, the PDWGF-triggered signal constitutes the activation of the MyD88/TRIF/MAPK/NF-κB pathway. Moreover, our results indicate that the combined action of TLR2 and TLR4 may be required for optimal induction of IL-1β in response to PDWGF. Thus, innate immune pathways, such as TLR2/4/MyD88/TRIF/MAPK/NF-κB and an NLRP3 inflammasome activation are involved in wheat proteins signaling and may play an important role in the pathogenesis of CD.

Introduction

Celiac disease (CD) is an inflammatory T cell-mediated disorder of the small intestine caused by the gluten fraction of wheat or the homologous proteins from barley and rye, in genetically predisposed individuals. An increased number of intraepithelial lymphocytes and lamina propria cells and their activation, followed by villous atrophy and crypt hyperplasia, characterize CD. Both innate and adaptive immune responses contribute to the onset of mucosal inflammation in CD patients [1]. Fragments of gliadin – a major group of proteins in gluten – cross the epithelium, and are presented by antigen presenting cells to the HLA-DQ2 or HLA-DQ8-restricted CD4+ α/β T lymphocytes present in jejunal mucosa [2]. Recently, other nongluten components of wheat (amylase inhibitors) were reported to

contribute to the specific response by stimulation of innate immunity cells [3]. The activated gliadin-specific T lymphocytes produce a spectrum of mediators and cytokines of a Th1 profile, mainly IFN-γ. These mechanisms could participate in intestinal tissue damage by contributing to the proinflammatory environment in the tissue and by activating tissue enzymes, including metalloproteases and tissue transglutaminase [4]. Besides IFN-γ, other cytokines such as IL-1β, IL-6, IL-15, IL-23 and TNF-α produced by innate immune cells contribute to the ongoing inflammation in CD [5–7].

IL-1β that belongs to the IL-1 cytokine family together with IL-1α, IL-18, and IL-33, has been associated with the inflammatory conditions in CD patients, and was shown to control the secretion of IL-23 leading to a shift to the Th1/Th17 immune pathway [7–9]. Production of IL-1β from inflammatory cells such as

monocytes and macrophages requires the following steps: the expression of the pro-IL-1β gene and the synthesis of immature pro-IL-1β protein; the cleavage of pro-IL-1β by active caspase-1 to yield the mature form of IL-1β; and the secretion of mature IL-1β from the cells. The generation of mature IL-1β is tightly controlled by a diverse class of cytosolic protein complexes, known as inflammasomes. Several different inflammasomes have been described, of which NLRP3 and NLRP1 (Nod-like receptor family, containing pyrin domain 3 and 1) inflammasomes have been the most intensively studied. Upon sensing danger signals, the NLRP3 proteins oligomerize and recruit caspase-1 through the adaptor protein apoptosis-associated speck like protein (ASC). Subsequently, caspase-1 undergoes an autocatalytic activation that involves the autoproteolytic processing of the 45-kDa pro-caspase-1 into 20- and 10-kDa subunits. In turn, mature caspase-1 cleaves pro-IL-1β, producing mature IL-1β [10].

In macrophages and dendritic cells (DCs), two temporally separate signals are required to yield the active proinflammatory cytokine. The first signal involves the activation of pattern recognition receptors [e.g. Toll like receptors (TLRs) or Nucleotide Oligomerization Domain (NOD)-like receptors] by pathogen- and danger-associated molecular patterns, which triggers the expression of pro-IL-1β via the NF-κB pathway [11]. Then microbial products [e.g. muramyl dipeptide [12], bacterial and viral RNA [13], and pore-forming toxins [14]] or endogenous signals, such as urate crystals [15] and adenosine triphosphate (ATP) [16] as the potential second signals, activate the NLRP3 inflammasome complex. Although the precise mechanism leading to the activation of NLRP3 remains largely unknown, it is proposed that oxidative stress, lysosomal destabilization with cytosolic cathepsin activity and potassium efflux due to the stimulation of ATP-sensitive potassium channels, or pore formation by bacterial toxins, converge into the activation of NLRP3 [17]. In human monocytes, contrary to the two-step signaling system in macrophages and DCs, differential requirements for the activation of the inflammasome were documented [18]. Caspase-1 is constitutively activated in these cells; therefore, a single stimulation event triggers the expression of pro-IL-1β and mature IL-1β release. The second signal is dispensable, because monocytes release endogenous ATP after stimulation, which in turn activate the inflammasome, and induces IL-1β secretion through the P2X7 receptor. IL-1β production is still dependent on the inflammasome components and modulated by K+ efflux [19,20].

In celiac patients, downstream products of NLRP3 inflammasome activation (such as IL-1β and IL-18) were shown to affect Th1/Th17 responses [7,21]. However, the mechanism of IL-1β activation has not yet been elucidated. Here, we analyzed the production of IL-1 cytokine family members in human monocytes and PBMC after stimulation with PDWGF, and investigated the upstream mechanism underlying PDWGF-induced IL-1β production and release in the PBMC of celiac patients. In particular, the role of the signaling molecules underlying de novo synthesis of pro-IL-1β [especially the role of TLRs, MyD88 and Toll-IL-1 receptor domain-containing adaptor-inducing interferon-β (TRIF); the role of MAPK JNK, ERK and p38 MAPK; the role of NF-κB and the mechanisms of caspase-1 activation culminating in IL-1β production] were studied.

Materials and Methods

Abs and Reagents
Glybenclamide, KN-62, N-Acetyl-L-cysteine (NAC), quinidine and polymyxin B were from Sigma-Aldrich (St. Louis, MO, USA). Benzyloxycarbonyl-Tyr-Val-Ala-Asp-(OMe) fluoromethylketone

(Z-YVAD-fmk) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). α-amylase inhibitor (AI) from Triticum aestivum type I and III were from Sigma. The p38 MAPK inhibitor SB203580, JNK inhibitor SP600125, serine-protease inhibitor N-p-Tosyl-L-phenyl-alanine chloromethyl ketone (TPCK) (all Sigma), and the ERK inhibitor UO126 (Cell Signaling Technology, Danvers, MA, USA) were dissolved in DMSO (Sigma). The FLICA Caspase-1 Assay kit was from ImmunoChemistry Technologies (Bloomington, MN, USA). Anti-human IL-1 β/IL-1F2 Ab and anti-mouse IL-1β/IL-1F2 Ab were from R&D Systems (Minneapolis, MN, USA), while anti-human cleaved IL-1β Ab was purchased from Cell Signaling Technology. Anti-caspase-1 (A19), anti-caspase-1 p10 (C20), and anti-beta actin were from Santa Cruz Biotechnology; anti-hTLR4-IgG(W7C11) and anti-hTLR2-IgA neutralizing Ab were from InvivoGen (San Diego, CA, USA). The following mAbs were obtained from BioLegend (San Diego, CA, USA): PE-Cy7-conjugated rat anti-mouse CD40 (3/23), BV-421-conjugated hamster anti-mouse CD80 (16-10A1), and Alexa Fluor488-conjugated rat anti-mouse CD86 (GL-1). Isotype control antibodies were from BD Biosciences (Mountain View, CA, USA) and eFluor 625NC-conjugated hamster anti-mouse CD11c (N418) was from eBioscience (San Diego, CA, USA).

Patients
In total, 81 subjects were enrolled in this study, including 39 symptomatic untreated patients with biopsy-proven celiac disease and 42 healthy controls. Men accounted for 48.2% of the patients and 51.8% of the controls. The mean age of the patients was 34.5 ± 8.9 years; the controls had a similar age distribution. Written informed consent was obtained from all patients and the study was approved by the Ethics Committee of the General Teaching Hospital, Prague.

Peptic Digest of Wheat Gliadin Fraction (PDWGF)
Peptic digest of wheat gliadin fraction (gliadin, Sigma-Aldrich, St. Louis, MO, USA) and ovalbumin (Hyglos, Bernried am Starnberger See, Germany) were prepared using pepsin agarose gel (MP Biomedicals, Illkirch Cedex, France) as described previously [22]. E-toxate test for LPS (Sigma) revealed that PDWGF contained less than 2 pg/ml endotoxin.

Isolation and Treatment of Monocytes and PBMC
PBMC and their fraction monocytes were isolated from the peripheral blood of CD patients and healthy volunteers, as described previously [23], and seeded in complete RPMI 1640 (Cambrex, East Rutherford, NJ, USA). PBMC or monocytes (2×10^5/400 µl) were incubated for 24 h in 24-well plates (Corning, Corning, NY, USA) in the presence of PDWGF (50, 100, 250 µg/ml). All reagents were tested by the E-toxate test for LPS (Sigma) and shown to be below the limit of detection (2 pg/ml). When indicated, Z-YVAD-fmk (10 µM), quinidine (100 µM), glybenclamide (100 µM), KN-62 (10 µM), NAC (30 mM), TPCK (25 µM), SP600125 (10 µM), SB203580 (20 µM), and UO125 (10 µM) were added 30 min before gliadin stimulation. KCl (50 mM) was used to increase extracellular K+ concentration. LPS (E. coli, 0111:B4, TLR grade; Alexis Biochemicals, Farmingdale, NY, USA) was used in concentration of 100 ng/ml. In some cases, anti-hTLR4 and anti-hTLR2 neutralizing Ab (10 µg/ml, Invivogen) were added 30 min before PDWGF stimulation. In experiments using PmB, PDWGF and/or LPS was incubated with 10 µg/ml PmB for 1 h at 4°C before addition to the cells. When indicated, PDWGF and AI were incubated with dithiothreitol (DTT, Sigma) and subsequently alkylated with iodacetamide (Sigma-

Aldrich) as previously described [3]. Reduced and alkylated (R/A) PDWGF and ATI were added to celiac PBMC in concentration of 100 µg/ml for 24 h. Cell culture supernatants were collected and stored at −80°C.

Mouse BMDC Preparation

WT C57BL/6 and C57BL/10, as well as mice deficient for NLRP3, ASC, IL-1R, MyD88 and TRIF (all on a C57BL/6 background) and mice deficient for TLR2, TLR4, and TLR2/4 (all on a C57BL/10 background), were bred under specific pathogen-free conditions in the animal facilities of the Max Planck Institute of Immunobiology and Epigenetics and the University Freiburg Medical Center. All of the experimental procedures were in accordance with institutional, state and federal guidelines on animal welfare and every effort was made to minimize suffering. The animal experiments were approved by the Regierungspräsidium Freiburg and supervised by the Animal Protection Representatives of the University Freiburg Medical Center or the MPI. Mice were anesthetized before sacrificing with 1% pelltobarbitalum natricum at the dose of 10 mg/kg. BMDC were prepared from bone marrow cells obtained from the femur and tibia in RPMI 1640 (Lonza, Basel, Switzerland) and 10% FBS (Cambrex), supplemented with 20 ng/ml GM-CSF from a GM-CSF expressing line. Cells (1×10^6 cells/ml) were washed and recultured with fresh medium containing 20 ng/ml GM-CSF every 3 d for 8 d. BMDC were cultured with PDWGF(100 µg/ml) for 21.5 h, and then 2 mM ATP (Sigma) was added for an additional 2.5 h. When indicated, Z-YVAD-fmk (10 µM) was added 30 min before PDWGF stimulation. Cells were used for flow cytometry analysis, or cell lysates were prepared and analyzed by western blot. Cell culture supernatants were analyzed by ELISA.

ELISA

The concentrations of human IL-1β, IL-1α, IL-18 and TNF-α as well as murine IL-1β and TNF-α were measured by commercial ELISA Duo Set Kits (R&D Systems) or ELISA MAX kits (Biolegend) according to manufacturer instructions.

FLICA Staining

Active caspase-1 was detected using the FLICA caspase-1 assay kit. Briefly, human PBMC (0.5×10^6 cells/0.5 ml) were treated with PDWGF (100 µg/ml) for 16 h prior to treatment with fluorescein-labeled inhibitor Z-YVAD-fmk (10 µM) for 1 h at 37°C. Cells were washed three times and analyzed by flow cytometry on a FACSCalibur (BD Biosciences), and the data were analyzed using CellQuest software (BD Biosciences).

Flow Cytometry

BMDC exposed to PDWGF, LPS, or OVA were stained with the relevant mAbs or isotype controls in a FACS bufer for 30 min on ice. In order to reduce non-specific Fc receptor-mediated binding, Fc block (CD16/CD32) from BD Biosciences was added to cells prior to and during staining. Cells were acquired on an LSR II flow cytometer (BD Biosciences) and DCs gated according to the FSC, SSC, and CD11c+ parameters for analysis.

Western Blotting

After treatment with Z-YVAD-fmk (10 µM), quinidine (100 µM), glybenclamide (100 µM), KN-62 (10 µM), KCl (50 mM), NAC (30 mM), TPCK (25 µM), SP600125 (10 µM), SB203580 (20 µM), and UO125 (10 µM) for 30 min, cells (1×10^6) were stimulated with PDWGF (100 µg/ml) for 24 h.

Cell supernatants were collected and cell lysates were prepared as previously described [24]. Cell lysates, as well as cell supernatants, were subjected to electrophoresis on a 5–20% SDS-PAGE gradient, and then transferred to nitrocelulose membranes for western blot analysis. After blocking with 5% fat-free dried milk for 1 h at room temperature, the membranes were incubated overnight with Abs raised against pro-IL-1β, cleaved IL-1β, caspase-1, and caspase-1p10. The membranes were revealed by HRP-conjugated secondary Ab (Cell Signaling Technology, Danvers, MA, USA) using the West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA). Western blot signals were detected using the LAS-1000 (Luminiscence Analyzing System; Fuji, Tokyo, Japan) and processed with AIDA 1000/1D image Analyzer software, version 3.28 (Raytest Isotopenmessgeraete, Straubenhardt, Germany). After stripping, the membranes were reprobed with anti-actin Ab (Abcam, Cambridge, USA).

DNA Extraction

Genomic DNA was extracted from the whole blood samples of CD patients and healthy donors using a standard salting out protocol [25]. Finally, total genomic DNA was quantified with a spectrophotometer, diluted at a final concentration 20 ng/µl and stored at −20°C.

Genotyping of NALP1 and NALP3 Polymorphisms

A single nucleotide polymorphism (SNP) in NALP1 (rs12150220) and NALP3 (rs10754558) was selected. Genotyping was performed with the 5′ nuclease assay technology for allelic discrimination using fluorogenic Taqman probes on a 7500 Fast Real Time system (Applied Biosystems, Foster City, CA, USA). Each SNP was analyzed by a specific Taqman SNP genotyping assay (Applied Biosystems). Briefly, the polymerase chain reaction (PCR) in 12 µl final volume with 20 ng of genomic DNA was performed using the ABI 7500 Thermal Cycler. The thermal cycle-sequencing conditions were as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturing at 95°C for 15 s and annealing and extension at 60°C for 1 min. Data were measured and analyzed using the Applied Biosystems Software Package SDS 2.1.

Statistical Analyses

Data were expressed as mean ± SD. Statistical analysis was performed using two-tailed Mann-Whitney U test from Graph Pad (PRISM 5.0). The statistical differences between the patient and the control groups for the genotyping of NALP1 and NALP3 polymorphism were analyzed by the Fisher's exact two-tailed test. Relative risk was estimated by calculating the odds ratio (OR) and 95% confidence interval (95% CI). We applied a Bonferroni correction for multiple comparisons in the analysis of variant allele distributions at each SNP. A value of $p < 0.05$ was considered to be statistically significant.

Results

PDWGF Induces IL-1β and IL-1α Release from Celiac Monocytes and PBMC

Gliadin digest, but not α-gliadin synthetic peptides, was found to stimulate IL-1β production in celiac PBMC and its monocytes fraction [7]. To investigate the underlying mechanism, we first determined the production of IL-1β, together with other members of IL-1 family, IL-18 and IL-1α. PBMC and monocytes from active CD patients and healthy donors were exposed to PDWGF (50, 100, 250 µg/ml). Doses of PDWGF

correspond to the expected concentrations of gliadin in the small intestine after a gluten-containing meal [26,27] and were used earlier in *in vitro* studies [7,28]. Subsequently, the secretion of IL-1β, IL-1α and IL-18 in culture supernatants was evaluated. Unstimulated monocytes and PBMC from healthy donors, as well as from celiac patients, spontaneously secreted negligible levels of IL-1β (Fig. 1A). Cells from healthy donors slightly increased IL-1β production upon PDWGF stimulation, while patients monocytes or PBMC strongly secreted (∼2-3-fold higher) IL-1β, even already when stimulated with 50 μg/ml. In contrast to robust IL-1β production, we found measurable but far lower levels of PDWGF-induced IL-18 in cells from healthy donors and from celiac patients (Fig. 1B). Next, we tested the production of IL-1α. Celiac PBMC and monocytes secreted significantly higher levels of IL-1α, in a dose-dependent manner after PDWGF treatment, when compared to the cells from healthy donors (Fig. 1C).

To exclude that the observed effect of PDWGF is due to LPS contamination, PDWGF was preincubated with PmB for neutral-ization of endotoxin. LPS preincubated with PmB was used as a control. As PmB was shown to directly trigger IL-1β secretion by activating the NLRP3 inflammasome [29], we tested the effect of PmB on PDWGF-induced TNF-α and IL-6 secretion. Preincuba-tion with PmB completely abrogated LPS-induced TNF-α and IL-6 production, but had no significant effect on PDWGF-induced TNF-α and IL-6 production by celiac PBMC (Fig. 1D).

PDWGF Induces IL-1β Production in a Caspase-1 Dependent Manner

To determine whether caspase-1 is required for PDWGF-induced IL-1β production, we used Z-YVAD-fmk, a specific caspase-1 inhibitor. PBMC were pretreated with Z-YVAD-fmk (10 μM) for 30 min and subsequently stimulated with PDWGF for 24 h. As shown in Fig. 2A, Z-YVAD-fmk reduced PDWGF-induced IL-1β production by 70±10% in PBMC from CD patients, but had no significant effect on PDWGF-induced IL-1α and TNF-α production.

We further confirmed that PDWGF-induced IL-1β production is caspase-1 dependent by western blot analysis. As shown in Fig. 2B, celiac PBMC treated with PDWGF for 24 h up regulated the expression of intracellular IL-1β, followed by the secretion and accumulation of mature IL-1β into supernatants. Caspase-1 inhibitor Z-YVAD-fmk strongly reduced the amount of the processed form of IL-1β in cell lysates, as well as supernatants, but had no effect on pro-IL-1β expression. In contrast, PDWGF stimulation of PBMC from healthy donors led to weak pro-IL-1β production with no detectable mature 17 kDa IL-1β form in cell lysates, nor cell culture supernatants.

We further showed that PDWGF induces processing of caspase-1 by the detection of an increased amount of the cleaved p10 subunit of caspase-1 in the conditioned supernatants from celiac, but not in healthy PBMC treated with PDWGF (Fig. 2C, D). PDWGF-induced elevated levels of caspase-1 subunit p10 were reduced in the presence of Z-YVAD-fmk. Finally, the activation of caspase-1 by PDWGF was determined by flow cytometry analysis, using a cell-permeable fluorescent probe that forms a covalent link with activated caspase-1. Our results revealed that PDWGF alone markedly increased caspase-1 activation in all celiac patients tested (mean increase of 212% ±73%, ranging from 52% to 515%). In healthy donors, the PDWGF-treated cells revealed a slight in-crease in caspase-1 activation (mean increase of 30% ±9%, ranging from 8% to 59%) compared to unstimulated cells (Fig. 2 E, F).
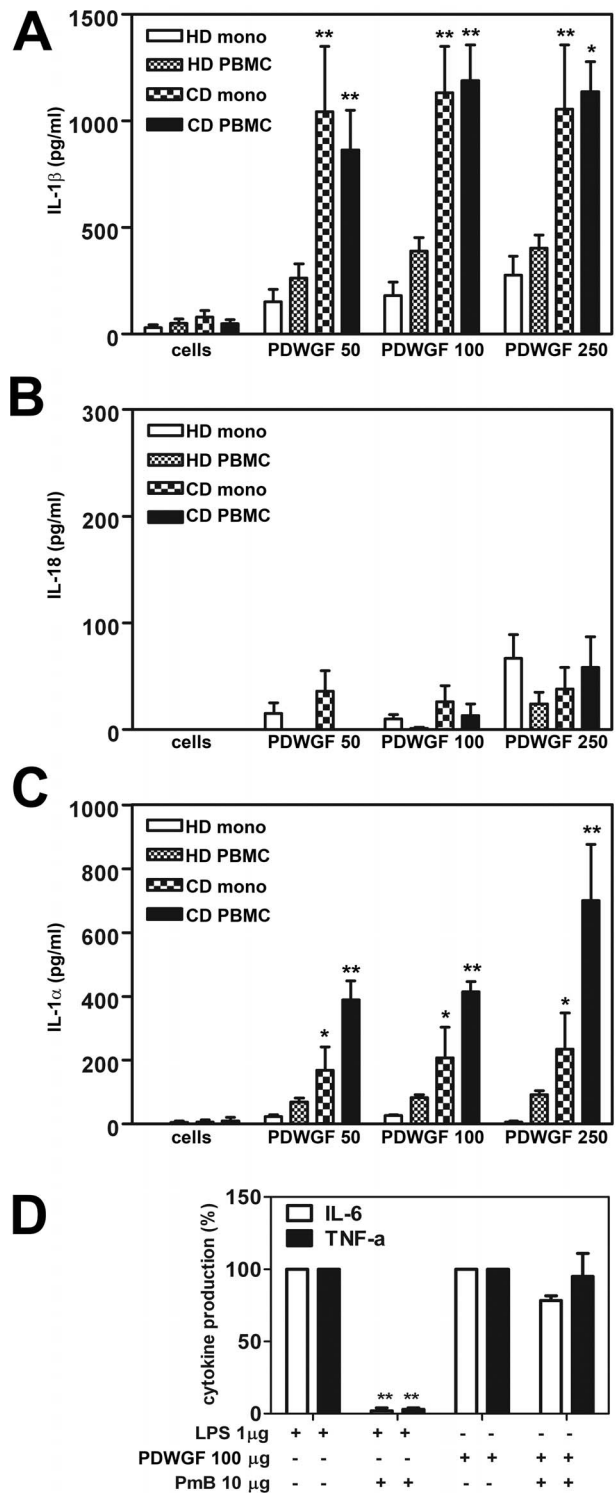

Figure 1. PDWGF digest induces IL-1β, IL-18, and IL-1α release in monocytes and PBMC from CD patients. IL-1β (A), IL-18 (B) and IL-1α (C) levels were quantified in cell supernatants by ELISA. Data are given as mean ± SD from 39 patients and 15 healthy donors (HD). *P<0.05, **P<0.01 (CD vs. HD). (**D**). PDWGF-induced activation of PBMC is not due to LPS contamination. Results are shown as the percentage of the cytokine production from 4 CD patients. The data were normalized to the result from untreated cells which was set as 100%. Mean ± SD, n = 4 independent experiments, ***P<0.001 compared to untreated cells.
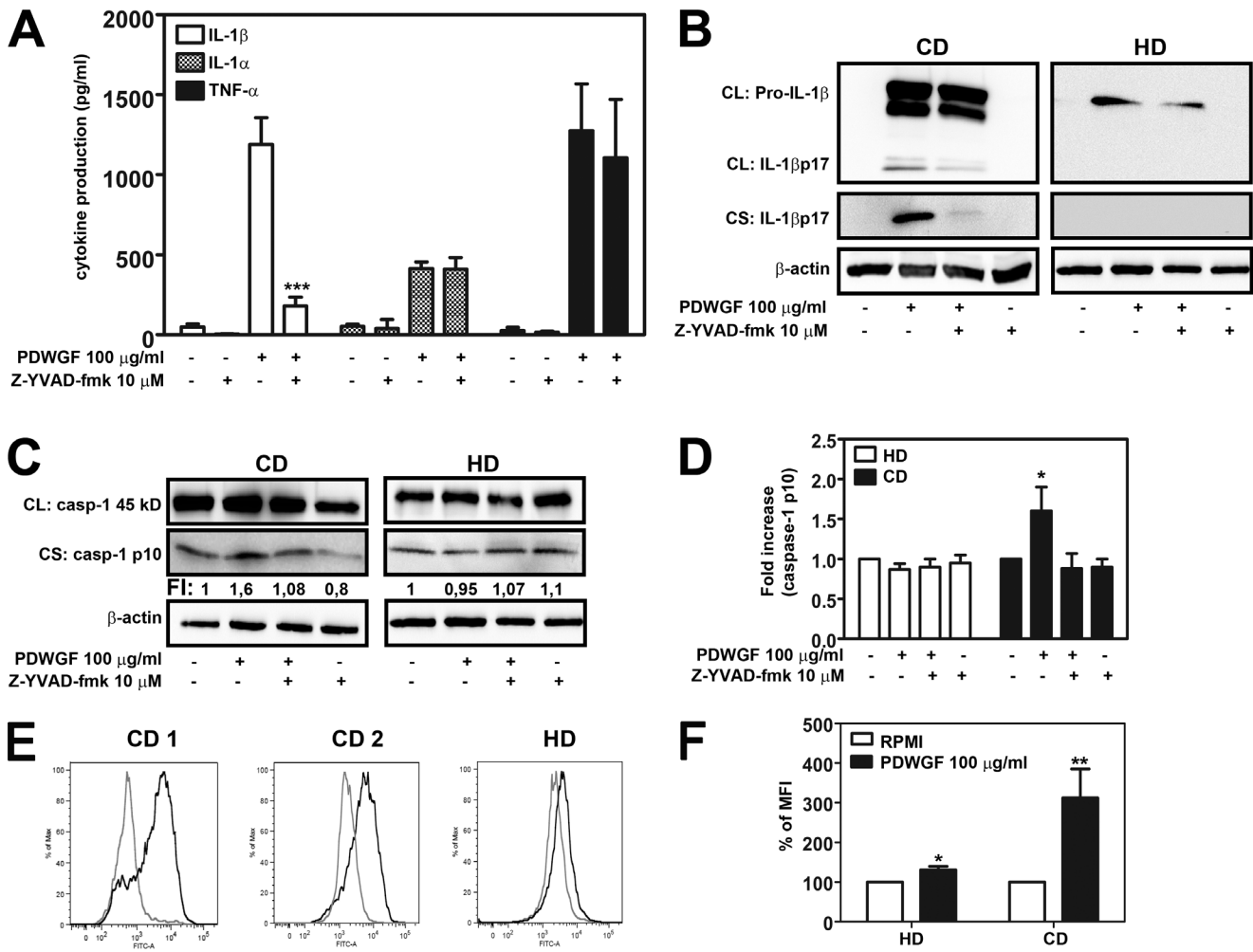
doi:10.1371/journal.pone.0062426.g001

Figure 2. The role of caspase-1 in PDWGF-treated PBMC. (**A**) Caspase-1 inhibitor Z-YVAD-fmk reduced PDWGF-induced IL-1β, but not IL-1α or TNF-α production. Mean ± SD, n = 10 independent experiments, ***P<0.001 compared to PDWGF-treated cells. (**B**) Western blot analysis for the expression of pro-IL-1β and mature IL-1β and (**C**) for caspase-1 and caspase-1 p10 in cell lysates (CL) and cell culture supernatants (CS) from PBMC. Representative blots from 5 independent experiments are shown. β-actin was used as a loading control. (**D**) The fold increase (FI) (densitometry analysis) of the quantity of caspase-1 p10 normalized to non-activated cells. Mean ± SD, n = 5 independent experiments, *P<0.05 compared to non-activated cells. (**E, F**) Direct activation of caspase-1 in PDWGF-treated PBMC, assessed by flow cytometry using a cell-permeable fluorescent probe. Results are shown as (**E**) a representative histogram from 2 CD patients and 1 HD; and (**F**) as the percentage of the MFI from 12 CD patients and 10 HD. The data were normalized to the result from untreated cells, which was set as 100%. Mean ± SD, *P<0.05 compared to untreated cells. CD, celiac disease patients; HD, healthy donors.
doi:10.1371/journal.pone.0062426.g002

PDWGF-induced IL-1β Secretion is Dependent on Potassium Efflux and ROS Production

To test if PDWGF-induced IL-1β secretion involves the potassium efflux from cells, we exposed PBMC to a medium containing 50 mM potassium chloride, which impedes potassium efflux. Under this condition IL-1β secretion was markedly reduced upon PDWGF stimulation (Fig. 3A). To confirm the effect of potassium efflux on IL-1β induction, we treated the cells with quinidine, a potassium channel inhibitor. Thus, we demonstrated that quinidine (100 μM) significantly decreased PDWGF-induced IL-1β production (Fig. 3A). When glybenclamide (100 μM) – another inhibitor of K+ efflux – was applied, a similar effect was seen. Next, we tested if PDWGF-induced IL-1β secretion required the P2X7 receptor. We used KN-62, a potent inhibitor of ATP-induced P2X7 receptor activation. Pretreatment of cells with KN-62 (10 μM) did not lead to a decrease of PDWGF-induced IL-1β production, suggesting that PDWGF may directly trigger potas-

sium efflux, bypassing the P2X7 receptor (Fig. 3A). Moreover, western blot analysis revealed that KCl, quinidine, and glybenclamide, but not KN-62, prevented IL-1β processing, as indicated by the failure to detect 17 kD IL-1β inside cells treated with PDWGF in combination with KCl or quinidine or glybenclamide (Fig. 3B).

Next, we tested if PDWGF might exert its effect on IL-1β production, not only by inducing K+ efflux, but also by inducing oxidative stress. PBMC were incubated for 30 min with ROS scavenger N-acetylcysteine (NAC) and stimulated with PDWGF for an additional 24 h. We found that PDWGF-induced mature IL-1β secretion detected by ELISA (Fig. 3C), as well as pro-IL-1β production detected by western blot (Fig. 3D), were markedly decreased when PBMC were stimulated with PDWGF digest combined with NAC; thus indicating that ROS may play a vital role in PDWGF-triggered IL-1β secretion (Fig. 3C, D).
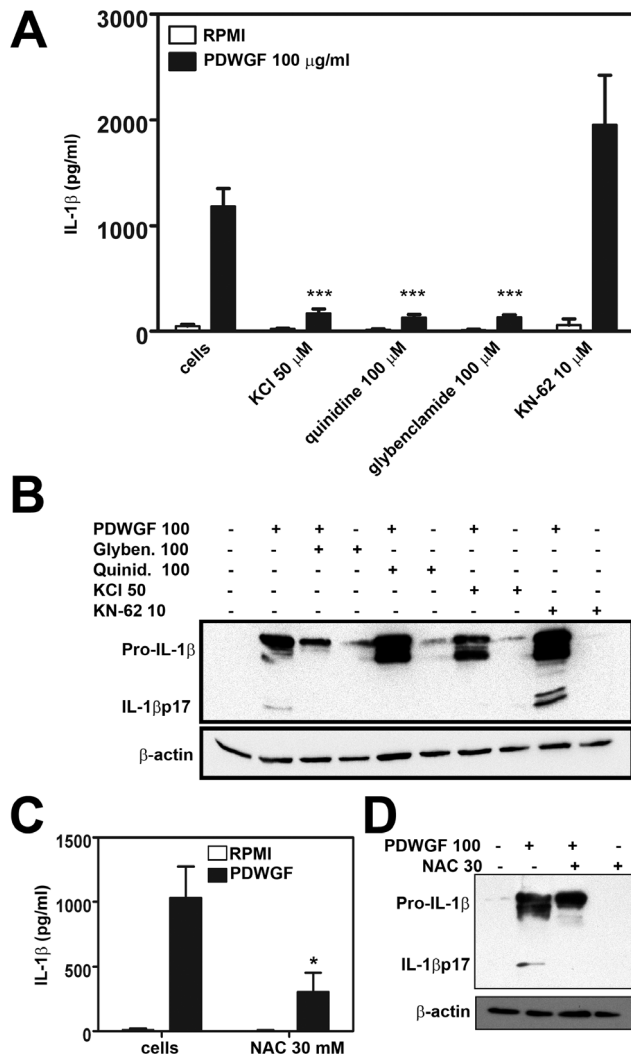
Figure 3. PDWGF-induced IL-1β production from celiac patient PBMC is modulated by K+ efflux, but is independent of the P2X7 receptor; as shown by (A) ELISA, mean ± SD, n = 10, ***P<0.001 vs. PDWGF-treated cells; and by (B) Western blot. Representative blots from 5 independent experiments are shown. (**C**) Inhibition of ROS modulate PDWGF-induced IL-1β secretion, mean ± SD, n = 10; as well as (D) pro-IL-1β production from PBMC of CD patients. Representative blots from 3 independent experiments are shown. β-actin was used as a loading control. ***P<0.001 vs. PDWGF-treated cells.
doi:10.1371/journal.pone.0062426.g003

The Genotype Frequencies of *NALP1 and NALP3* Polymorphism Among Celiac Patients and Control Subjects

As recent studies suggested the important role of NALP1 and NALP3 genes in the predisposition to autoimmune disorders, we evaluated the possible association of single nucleotide polymorphisms (SNPs) in NLRP1 and NLRP3 genes in celiac patients and in healthy individuals. Our data (Table 1) show a significant difference in the frequencies of the SNP rs10754558 GG NALP3 genotype between the controls and patients. These data suggest that the GG genotype of NALP3 gene could play a protective role in coeliac disease (the patients group N = 1, 2.5%; the control group N = 9, 21.4%). Assessment of NALP1 and NALP3 genotype

combinations in patients and control does not confirm any statistical difference between these two groups (data not shown).

PDWGF Digest Induces Pro-IL-1β Synthesis via the MAPK-NF-κB Pathway

Next, we analyzed whether the NF-κB signaling pathway is involved in PDWGF-induced IL-1β production. Celiac PBMC were pretreated with serine protease inhibitor TPCK (25 μM) for 30 min and subsequently stimulated with PDWGF (100 μg/ml) for 24 h. Treatment with TPCK completely abolished PDWGF-induced IL-1β production (Fig. 4A), as well as all synthesis of pro-IL-1β (Fig. 4B) after PDWGF stimulation, indicating that NF-κB is a critical player during PDWGF-induced processes leading to pro-IL-1β synthesis and IL-1β release.

Furthermore, we tested if PDWGF-mediated phosphorylation of MAPKs is an upstream event leading to *de novo* synthesis of pro-IL-1β. PBMC were pre-treated with an inhibitor of p38 MAPK SB203580 (20 μM), an inhibitor of JNK SP600125 (10 μM), and an inhibitor of ERK UO125 (10 μM) for 30 min and then stimulated with PDWGF for an additional 24 h.

We found that PBMC from CD patients treated with PDWGF in combination with every single MAPK inhibitor displayed markedly reduced IL-1β secretion, when compared to cells treated with PDWGF alone (Fig. 4A). Moreover, the markedly reduced synthesis of PDWGF-induced pro-IL-1β in PBMCs treated with PDWGF combined with every single inhibitor tested, was confirmed by western blot analysis (Fig. 4B).

PDWGF Stimulates Secretion of IL-β in Mouse BMDC in a Caspase-1 Dependent Manner and Requires NLRP3 and ASC

Our studies showing that PDWGF digest stimulates caspase-1 dependent production of IL-1β in celiac PBMC, were further extended to BMDC from mice. To analyze the role of the NLRP3 inflammasome in PDWGF-induced IL-1β release, BMDC from WT C57BL6 mice, and NLRP3−/− and ASC−/− KO mice were used. Since exogenous ATP was shown to be required for the production of mature IL-1β in macrophages and DC stimulated with TLR ligands [30], BMDC were treated with PDWGF for 21.5 h; subsequently 2 mM ATP was added for an additional 2.5 h; and IL-1β production was then determined in cell culture supernatants by ELISA. As shown in Fig. 5A, the stimulation of WT BMDC with PDWGF led to increased IL-1β secretion that was markedly elevated when exogenous ATP was added. In contrast, PDWGF and ATP-stimulated BMDC – deficient in NLRP3 or ASC proteins – secreted very low levels of IL-1β compared to the WT BMDC. On the other hand, NLRP3 and ASC molecules were dispensable for the PDWGF-induced production of TNF-α (data not shown), indicating an unimpaired cytokine production capacity of BMDC from these mouse strains. Additionally, in contrast to the induction of IL-1β, flow cytometric analysis of maturation markers (CD40, CD80, CD86) on BMDC revealed that PDWGF and LPS (as positive control) induced a similar increased maturation of BMDC, and that this effect was not dependent on the inflammasome component NLRP3 (Fig. 5B). Moreover, we found that, similar to human PBMC, treatment of mouse BMDC with caspase-1 inhibitor Z-YVAD-fmk resulted in a marked decrease of IL-1β production from PDWGF and ATP-treated cells (Fig. 5C). These data suggest that PDWGF digest induces IL-1β production through the NLRP3 inflammasome complex, and that caspase-1 is necessary for IL-1β secretion in BMDC.

Table 1. Genotype frequencies for *NALP1* (rs12150220) and *NALP3* (rs10754558).

	Genotype/	Control (%)	Case (%)	P-value	OR	95% CI
	Allele	**n (42)**	**n (39)**			
rs 12150220	AA	13 (30.9%)	10 (25.6%)	0.6252	ns	ns
	AT	19 (45.2%)	15 (38.5%)	0.5052	ns	ns
	TT	10 (23.9%)	14 (35.9%)	0.4962	ns	ns
	A	45 (40.5%)	35 (44.9%)	0.2764	1.418	0.7634–2.6333
	T	39 (46.5%)	43 (55.1%)			
rs 10754558	CC	17 (40.5%)	16 (41.0%)	1.0000	ns	ns
	CG	16 (38.1%)	22 (56.4%)	0.2573	ns	ns
	GG	9 (21.4%)	1 (2.5%)	**0.0141**	**0.0877**	**0.01501–0.731**
	C	50 (59.5%)	54 (69.2%)	0.2510	0.6536	0.3415–1.251
	G	34 (40.5%)	24 (30.8%)			

OR- odds ratio, CI- confidence interval.
doi:10.1371/journal.pone.0062426.t001

PDWGF Stimulates BMDC to IL-1β Production through TLR, MyD88 and TRIF

Our data showing the involvement of MAPKs JNK, ERK, and p38, and of the NF-κB signaling pathway in PDWGF-induced IL-1β production from celiac PBMC (Fig. 4), led us to study upstream TLR signaling. First, we assessed the role of the MyD88 adaptor molecules, as well as TRIF, in the induction of IL-1β by PDWGF. BMDC from C57BL10 WT, MyD88, and TRIF KO mice were stimulated with PDWGF (100 μg/ml) and ATP (2 mM), as

Figure 4. MAPK and NF-κB are involved in PDWGF mediated IL-1β secretion. PDWGF-primed PBMC were treated with SB203580 (SB), SP600125 (SP), UO125 (UO), TPCK, or PDWGF, alone or in combination for 24 h. (**A**) IL-1β was quantified in cell supernatants by ELISA. Mean ± SD, n = 5 independent experiments. ***P<0.001 vs. PDWGF-treated cells. (**B**) Pro-IL-1β levels were examined in cell lysates by immunoblotting from 5 experiments. β-actin was used as a loading control.
doi:10.1371/journal.pone.0062426.g004

described above. We found that the secretion of IL-1β induced by PDWGF alone or in combination with ATP, was significantly reduced in MyD88 and TRIF-deficient BMDC (Fig. 6A). Consistently, the induction of pro-IL-1β in response to PDWGF was abrogated in MyD88 KO BMDC and markedly reduced in TRIF KO BMDC (Fig. 6B). In contrast, the induction and secretion of IL-1β was not reduced in BMDC deficient in IL-1R (Fig. 6B). These results suggest that TLR signaling is essential for pro-IL-1β induction in response to PDWGF digest.

By analyzing PDWGF-induced IL-1β secretion in TLR2, TLR4, and TLR2/4 KO mice, we found that the secretion of IL-1β, induced by PDWGF, alone or in combination with ATP, was significantly reduced in TLR4 KO BMDC and abrogated in BMDC from mice deficient for both TLR2 and TLR4 (Fig. 6C). Consistently, the induction of pro-IL-1β in response to PDWGF was significantly reduced in BMDC deficient in TLR4, but not TLR2 (Fig. 6D). On the other hand, BMDC from mice deficient for both TLR2 and TLR4 displayed completely abrogated pro-IL-1β production after stimulation with PDWGF, suggesting some additional role of TLR2. The findings on PDWGF-induced pro-IL-1β and IL-1β production in mouse BMDC were further confirmed in a human system, as anti-TLR4 mAb substantially reduced PDWGF-induced IL-1β release by 70% in celiac PBMC, while anti-TLR2 mAb revealed no effect on PDWGF-induced IL-1β release (Fig. 6E).

Very recently, Junker *et. al.* [3] reported that nongluten wheat amylase inhibitors (AI) that copurify with ω-gliadins are present in gliadin digest and can stimulate IL-8 cytokine production via TLR4 pathway. Moreover, the highly disulfide-linked secondary structure of AI is necessary to activate TLR4. Thus, we tested whether wheat AI are able to stimulate IL-1β secretion in our system. We found that AI stimulated celiac PBMC to robust secretion of IL-1β, comparable to those induced by PDWGF (Fig. 7). Next, we evaluated whether reduction and alkylation of AI as well as PDWGF will affect the capacity to induce IL-1β secretion. We found that reduction and alkylation of AI as well as PDWGF completely abolished IL-1β secretion from celiac PBMC (Fig. 7).

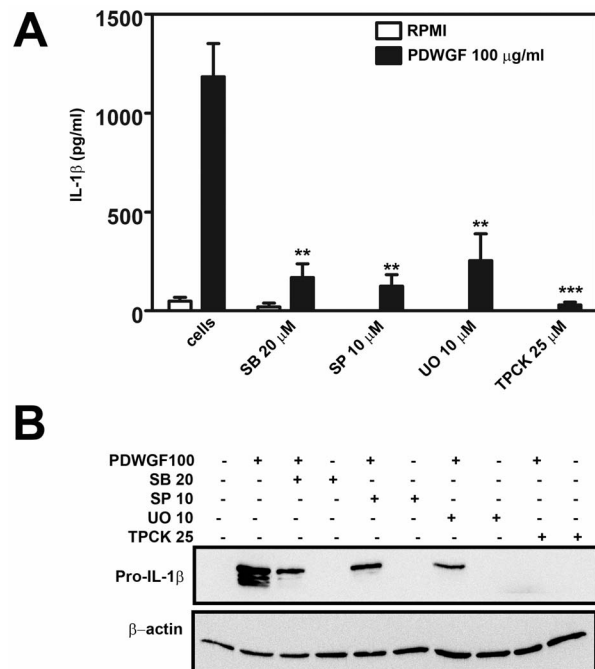## Discussion

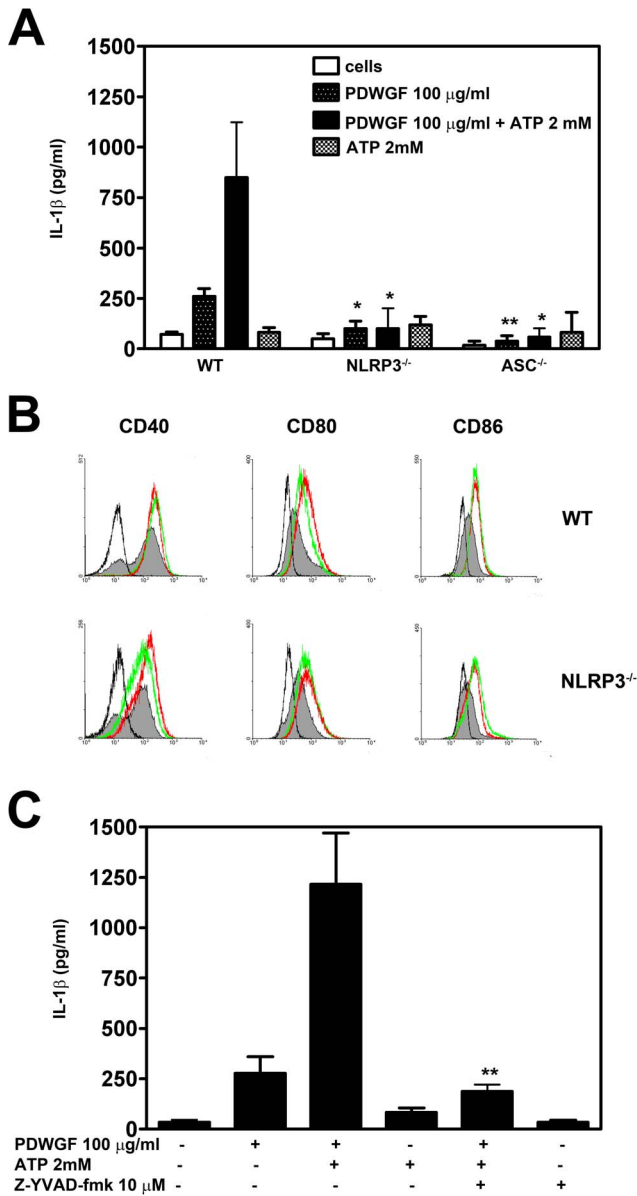We have shown here, in line with previous study [7], that PDWGF is capable of inducing robust IL-1β production by

Figure 5. PDWGF stimulates BMDC to IL-1β production through NLRP3 and ASC. (**A**) BMDC from WT, NLRP3−/− and ASC−/− mice were exposed to PDWGF (100 μg/ml) alone for 24 h; or first PDWGF was added for 21.5 h, the subsequently ATP (2 mM) was added for additional 2.5 h. IL-1β was measured in culture supernatants. (**B**) Flow-cytometric evaluation of PDWGF-induced maturation assessed by CD40, CD80, and CD86 expression on BMDC from WT and NLRP3−/− mice. WT and NLRP3−/− BMDC were cultured with 100 μg/ml of PDWGF (green), as well as 0.1 μg/ml of LPS (red) or 100 μg/ml of OVA (grey-filled) as positive and negative controls, respectively. Isotype controls are represented in black overlays. (**C**) Cells were preincubated with caspase-1 inhibitor Z-YVAD-fmk for 30 min, and then exposed to PDWGF in combination with ATP. Production of IL-1β was measured in culture supernatants. Results are expressed as mean ± SD from 4 independent experiments. The levels of significance for KO BMDC vs. WT BMDC are indicated as follows: *P<0.05, **P<0.01, and ***P<0.001. doi:10.1371/journal.pone.0062426.g005

monocytes and PBMC in celiac patients. Moreover, we show for the first time that PDWGF induces significantly increased amounts of IL-1α from monocytes and PBMC in celiac patients, and slightly elevated amounts of IL-18.
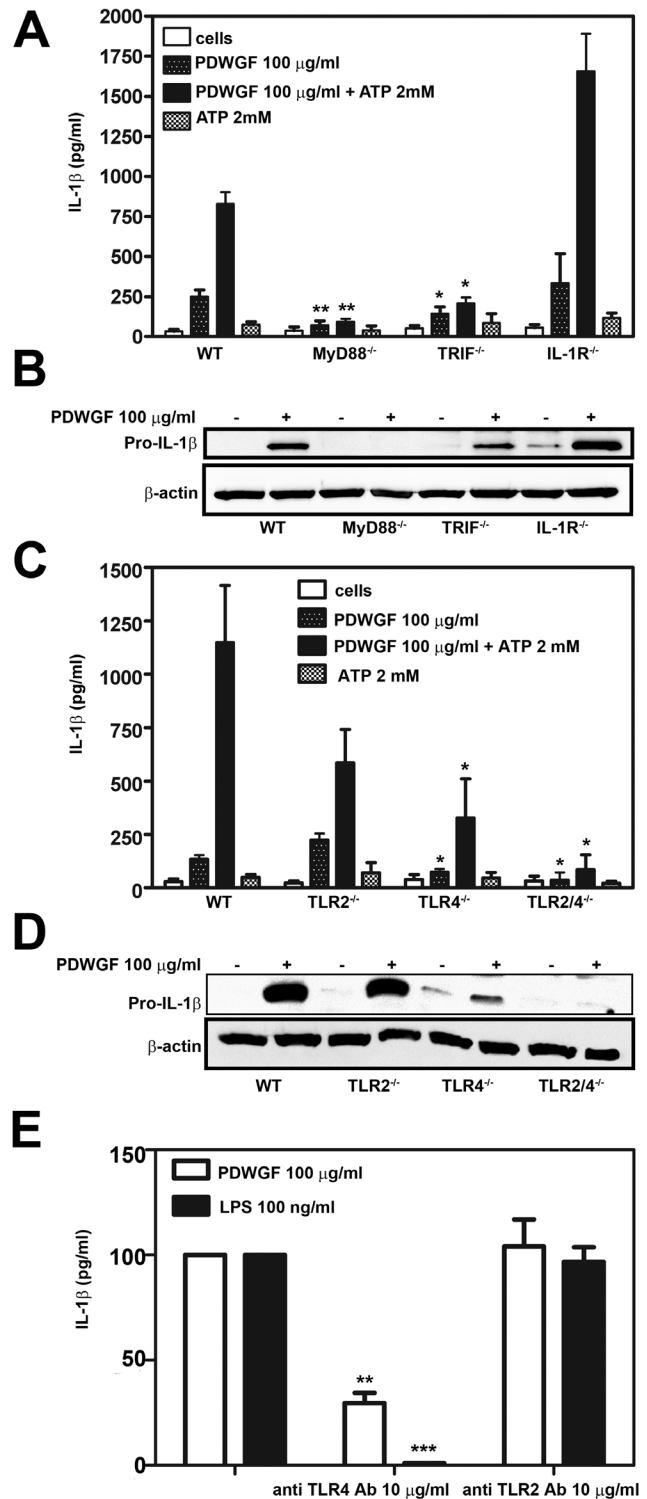
Figure 6. TLR signaling is required for pro-IL-1β synthesis in response to PDWGF. WT BMDC and MyD88−/−, TRIF−/−, and IL-1R−/− KO BMDC were treated with PDWGF alone or in combination with ATP and (**A**) IL-1β production was evaluated after 24 h. (**B**) Cell lysates were evaluated for *de novo* synthesis of pro-IL-1β (**C**) WT BMDC and TLR2−/−, TLR4−/−, and TLR2/4−/− KO BMDC were treated with PDWGF alone or in combination with ATP, and IL-1β production was evaluated after 24 h. (**D**) Cell lysates were evaluated for *de novo* synthesis of pro-IL-1β. Data in (A) and (C) are expressed as mean ± SD from 5 independent experiments. *P<0.05, **P<0.01 vs. WT BMDC. Blots in (B) and (D) are representative from 3 independent experiments.

β-actin was used as a loading control. (**E**) Celiac PBMC were treated with PDWGF alone or in combination with anti-TLR4 or anti-TLR2 Ab. IL-1β secretion was evaluated after 24 h. LPS was used as a positive control. Mean ± SD, 8 independent experiments. **P<0.01, ***P<0.001 vs. cells without anti-TLR Ab.
doi:10.1371/journal.pone.0062426.g006

Next, we investigated the molecular mechanisms underlying the PDWGF-induced production of IL-1β in celiac PBMC. In this study we clearly document that PDWGF-induced IL-1β production by celiac PBMC is caspase-1 dependent. Interestingly, we observed that active caspase-1 was already present in unstimulated PBMC and PDWGF was able to markedly increase caspase-1 activation and the processing of pro-IL-1β in celiac PBMC, in contrast to those of healthy donors. Our data correlate with prior findings that celiac patients display the active form of caspase-1 and mature IL-18 protein in small bowel mucosa [31]. Moreover, we have confirmed that PDWGF-induced IL-1β release was dependent on NLRP3 and ASC, as shown by the stimulation of $NLRP3^{-/-}$ and $ASC^{-/-}$ BMDC. The role of the NLRP molecule in the predisposition to, or progression of CD remains unclear. Our data propose that the GG genotype of the SNP rs10754558 NALP3 gene could play a protective role in celiac disease. Interestingly, a similar trend has been seen in the first study of Pontillo et al. [32], where the G allele was protective against the development of CD (non-significantly), but this effect has not been confirmed in their following study [33]. However, similarly with our observation, neither study has found any NALP1 allele/genotype association with autoimmune disease.

Next, our study illustrated that IL-1β production from celiac PBMC is dependent on K+ efflux, since pro-IL-1β processing, as well as secretion of IL-1β were reduced when K+ efflux was impeded. In human blood monocytes, K+ efflux is mediated through membrane pores formed by P2X7 molecules after activation by autocrine released endogenous ATP [18]. Surprisingly, our data did not confirm the role of the P2X7 receptor in gliadin-induced IL-1β production in celiac PBMC, since KN-62 did not reduce either processing or secretion of gliadin-induced IL-1β. This conflict can be explained by a two step interleukin IL-1β secretion from human PBMC through both P2X7-dependent, as well as P2X7-independent mechanisms [34]. Our data show that prolonged (24 h) exposure to PDWGF, leading to elevated IL-1β secretion, does not involve the P2X7 receptor. When we tested PDWGF-induced IL-1β production after 3 h, the levels of IL-1β were below the detection limit, and we could not test the effect of KN-62. On the contrary, KN-62 markedly reduced the release of IL-1β by PBMC primed for 3 h by PDWGF and then treated with ATP for 30 min. This data suggests that the early release of PDWGF-induced IL-1β might be mediated via P2X7 receptor (data not shown).

We have further demonstrated that NF-κB is a critical player during the PDWGF-induced processes leading to pro-IL-1β synthesis and IL-1β release in celiac PBMC. Moreover, we show that in addition to NF-κB, MAPK pathways orchestrate PDWGF-induced de novo synthesis of pro-IL-1β in celiac cells. These data are in line with our previous studies showing that pepsin digest of gliadin triggers the activation and maturation of human DCs and PBMC via NF-κB and the p38 MAPK pathway [23,35].

The nature of the receptors engaged by gliadin digests as well as gliadin peptides (or other wheat components) responsible for mediating innate immunity remains to be clearly identified. Gliadin is a complex protein containing multiple epitopes that exert cytotoxic, immunomodulatory and intestinal permeability effects. Some gluten-derived peptides elicit T-cell specific re-
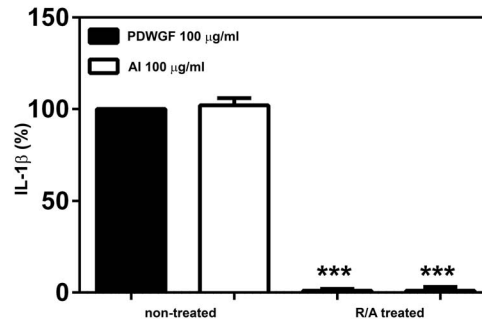

Figure 7. Reduction and alkylation (R/A) of PDWGF led to abrogated IL-1β production. PDWGF as well as α-amylase inhibitor (non-treated or R/A treated) were added to celiac PBMC. IL-1β secretion was evaluated after 24 h. Results are shown as the percentage of the cytokine production from 5 CD patients. The data were normalized to the result from PDWGF-treated cells which was set as 100%. Mean ± SD, 5 independent experiments. ***P<0.001 vs. non-treated PDWGF.
doi:10.1371/journal.pone.0062426.g007

sponses, while some other "non-immunodominant" gliadin derived peptides stimulate innate immunity response in the gut [36–39]. The chemokine receptor CXCR3 was shown to serve as a receptor for specific gliadin peptides that cause zonulin release and subsequent increase in intestinal permeability [40]. Next, MyD88 was shown to be involved in gliadin-induced inflammatory cytokine production in mouse macrophages [6]. In our study, PDWGF-induced IL-1β production was strongly dependent on MyD88, and to certain extend on TRIF, which suggests that components of gliadin digest could induce signaling via TLR receptors. As LPS contamination was ruled out in our study, it seems that the stimulatory effect of our gliadin preparation is caused by proteins. In mouse models, TLR2 and TLR4 were not identified as a component of gluten or gliadin-induced signaling pathway [6,41]. On the other hand, very recently, Junker et al., [3] reported that activation of innate immune responses by pepsin trypsin gliadin digest is due to wheat amylase trypsin inhibitors CM3 and 0.19 that signal via TLR4/CD14 complex. Moreover, the highly-disulfide linked secondary structure of amylase trypsin inhibitors is very important for activation of TLR4. Here, we documented that reduction of our PDWGF led to completely abrogated capacity to stimulate IL-1β production in PBMC. It suggests that amylase trypsin inhibitors may be present in our PDWGF and they may orchestrate in IL-1β production. We also documented that BMDC from TLR2−/− mice produced slightly reduced IL-1β levels, whereas BMDC from TLR4−/− mice displayed significantly decreased IL-1β levels after the PDWGF triggering. Interestingly, cells deficient for both TLR2 and TLR4 displayed completely abrogated IL-1β production, indicating that the combined action of TLR2 and TLR4 may be involved in PDWGF signaling. Notably, the combined action of the two TLRs was shown to be required for sensitization to contact allergens in a murine model of allergic contact dermatitis [42], and for the regulation and tissue repair in bleomycin-induced mouse tissue injury [43]. Our murine data, suggesting a role of TLR in the signaling pathway underlying IL-1β production, was confirmed in humans using anti-TLR4 Ab that markedly reduced PDWGF-induced IL-1β in celiac PBMC. The possible role of TLR involvement is supported by the fact that celiac patients were found to have higher expression of TLR2 and TLR4 molecules in intestinal tissue [44]. Moreover, they exhibited a higher prevalence of TLR2 and TLR4 positive blood DCs and monocytes compared to controls [45]. In contrast, recently other group [46] reported

decreased levels of TLR receptors in the small intestinal biopsies of celiac patients.

However, as we did not observe a complete inhibition of TLR signaling using the anti-TLR4 antibody in PBMC, it is plausible that also here TLR2 and/or other signaling molecules may be involved in the activation of human cells in response to PDWGF.

In conclusion, in accordance to recently published study [3], our findings suggest that PDWGF does not only contain T cell epitopes, but also other wheat components that could play a role as adjuvants activating the innate immune system via TLR2/4, ROS, and the NLRP3 inflammasome. In addition, T cell and NK cell responses through IFN-γ were shown to strongly potentiate the pro-inflammatory activity of TLR ligands [47]. Both mechanisms may be involved in the pathogenesis of wheat allergy as well as CD.

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3.2.2. Publication VII.

Funda DP, **Fundová P**, Hansen AK, Buschard K. Prevention or Early Cure of Type 1 Diabetes by Intranasal Administration of Gliadin in NOD Mice. ***PLoS One*, 2014; 9(4):e94530.** IF (2015) = 3.057

Dietary gluten or wheat proteins are among known environmental factors that modify type 1 diabetes incidence in the spontaneous animal models (Scot, 1996; Funda et al., 1999; Hansen et al., 2014). Interestingly, the increased association of T1D and celiac disease points not only to the shared risk HLA antigens but also to the diet, as patients diagnosed with both celiac disease and T1D usually develop diabetes first and not vice versa (Cosnes et al., 2008), suggesting beneficial effect of gluten-free diet also in humans. Several human trials with intranasal or oral administration T1D autoantigens such as insulin or GAD65 have so far failed to show protective effects (Hanninan and Harrison, 2004; Fousteri et al., 2007).

In this study we explored whether, mucosal, intranasal administration of gliadin or gluten may prevent development of diabetes in NOD mice. We showed that i.n. administration of gliadin, but not gluten, at age of 4 weeks led to significant decrease of diabetes incidence in NOD mice fed a standard gluten-containing diet. Interestingly, this intervention was less but still statistically significantly preventing diabetes when applied to 13-week-old NOD mice, just before clinical manifestation of the disease. Following the i.n. administration of gliadin or OVA we then assessed proportions of Tregs and their cytokine signatures in both mucosal (NALT, MLN, PLN) and control, systemic lymphoid organs (spleen, inguinal lymph nodes). There was a clear mucosal induction - at the site of the application, i.e. in the NALT, and in the MLNs and PLNs, of CD4$^+$Foxp3$^+$ T cells and even more significant increase of γδ T cells after i.n. gliadin administration. This distribution well corresponds with the concept of the common mucosal immune system (Mestecky, 1987). Increased induction of IL-10 and decreased proportion of IL-2, IL-4 and IFN-γ in CD4$^+$Foxp3$^+$ Tregs, as well as decreased proportion of IFN- γ in γδ T cells was observed after i.n. gliadin in preferentially mucosal lymphoid organs.

Interestingly, there is evidence for regulatory role of mucosal γδ T cells in T1D as intranasal aerosol application of the whole insulin molecule also led to induction of CD8+ γδ T cells that were capable of preventing T1D in the adoptive co-transfer model (Harrison et al., 1996). We have also shown increased proportion of mucosal γδ T cells in relation to the effect of the diabetes-preventive, gluten-free diet (Antvorskov et al., 2012).

The effect of i.n. gliadin is not however due to induction of specific tolerance. The NOD mice were fed gluten containing diet and at 4 weeks of age have already had well-established oral tolerance to gliadin, as even short or single oral exposure is sufficient for induction of oral tolerance (Strobel and Mowat, 1998). We suggest that perhaps innate immune properties of gliadin may be responsible for the diabetes preventive effect. Similar to prolonged or no exposure to LPS, both gluten-free and gluten-enriched diets prevented diabetes in NOD mice (Funda et al., 2008). In conclusion, intranasal application of gliadin, an environmental antigen that may have an etilogocal influence in T1D, represent a novel and safe approach for prevention or even early cure of T1D.

Prevention or Early Cure of Type 1 Diabetes by Intranasal Administration of Gliadin in NOD Mice

David P. Funda[1,2]*, Petra Fundova[1,2,3], Axel Kornerup Hansen[4], Karsten Buschard[1]

1 The Bartholin Instituttet, Rigshospitalet, Copenhagen, Denmark, 2 Department of Immunology and Gnotobiology, Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, Prague, Czech Republic, 3 ENT Department of the 3rd Faculty of Medicine, Charles University and the Central Military Hospital, Prague, Czech Republic, 4 Section of Biomedicine, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark

Abstract

Induction of long-term tolerance to β-cell autoantigens has been investigated both in animal models and in human type 1 diabetes (T1D) in order to prevent the disease. As regards external compounds, the dietary plant protein fraction has been associated with high penetrance of the disease, whereas gluten-free diets prevent T1D in animal models. Herewith we investigated whether intranasal (i.n.) administration of gliadin or gluten may arrest the diabetogenic process. I.n. administration of gliadin to 4-week-old NOD mice significantly reduced the diabetes incidence. Similarly, the insulitis was lowered. Intranasal gliadin also rescued a fraction of prediabetic 13-week-old NOD mice from progressing to clinical onset of diabetes compared to OVA-treated controls. Vaccination with i.n. gliadin led to an induction of CD4$^+$Foxp3$^+$ T cells and even more significant induction of γδ T cells in mucosal, but not in non-mucosal lymphoid compartments. This prevention strategy was characterized by an increased proportion of IL-10 and a decreased proportion of IL-2, IL-4 and IFN-γ-positive CD4$^+$Foxp3$^+$ T cells, and IFN-γ-positive γδ T cells, preferentially in mucosal lymphoid organs. In conclusion, i.n. vaccination with gliadin, an environmental antigen with possible etiological influence in T1D, may represent a novel, safer strategy for prevention or even early cure of T1D.

Introduction

The incidence of type 1 diabetes mellitus (T1D) has been rapidly increasing during the past decades [1]. In humans, the autoimmune process is prolonged as clinical onset of the disease does not occur until approximately 80% or more β cells are destroyed [2] leaving a time window of opportunity for therapeutical or preventive intervention in prediabetic individuals. In animal models of T1D, mucosal administration of β-cell related autoantigens is a well-established strategy for disease prevention by induction of islet-specific T regulatory cells (Tregs) that may prevent the autoimmune aggression locally by mechanism of bystander suppression [3]. Several β-cell autoantigens, defined rather by occurrence of autoantibodies than T cell specific immunoreactivity exclusive for T1D patients, have been used for prevention of T1D by mucosal (oral, intranasal) administration. Mucosal administration of insulin [4,5] or GAD [6], GAD65 peptide [7] as well as proinsulin or insulin peptides [8,9] has led to prevention of T1D in animal models. Several of the autoantigens have been used in recent human trials, but at present, there is no established prevention strategy available for human T1D [10,11].

Studies in both NOD mice and BB rats have documented that T1D is a diet-influenced disease. Wheat flour is an essential component of diabetes-permissive, non-purified diets and purified diets based on hydrolyzed casein, lactalbumin or amino acids

prevented development of diabetes in NOD mice and BB rats [12–14]. We and others have documented that a non-purified, gluten-free diet highly prevents development of diabetes in NOD mice [15,16].

The diabetogenic role of gliadin is also implicated by a more frequent clinical association of T1D and celiac disease. Although celiac disease and T1D share similar risk HLA antigens, patients diagnosed with both celiac disease and T1D usually develop diabetes first and not vice versa [17]. Patients with celiac disease have an earlier onset of T1D [18] and there is also one report of enhanced T reactivity to gluten in newly diagnosed type 1 diabetics [19]. Early introduction of dietary gluten was reported to increase the risk of developing islet autoantibodies in children [20]. Gluten-free diet also induces changes in the gut microbiota of NOD mice [21]. These observations suggest an etiological role for gliadin in T1D.

Using the NOD mouse model, we investigated the effect of intranasal (i.n.) administration of gluten or gliadin at the age of 4 weeks on development of insulitis and clinical onset of diabetes. We tested whether i.n. vaccination may rescue animals from developing diabetes when applied just before clinical onset of diabetes at the age of 13 weeks. We also investigated whether this i.n. prevention strategy leads to induction of potentially regulatory T cells and changes in their cytokine profiles.
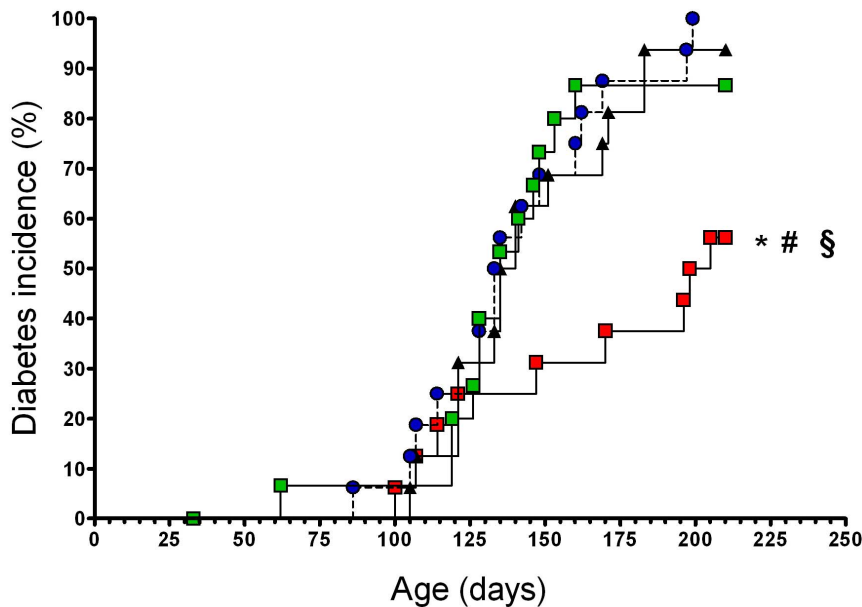
Figure 1. Intranasal (i.n.) administration of gliadin decreases diabetes incidence in NOD mice. A statistically significant decrease in diabetes incidence was found in NOD mice treated with gliadin (red square) compared to OVA (blue circle), *p = 0.001 or PBS (black triangle), #p = 0.008 controls, whereas no diabetes-protective effect was found in gluten-treated (green square) group. The gliadin-treated group displayed decreased diabetes incidence (§p = 0.029) also compared to gluten-treated (closed circle) NOD mice. Results are representative of three or two (gluten) independent experiments.
doi:10.1371/journal.pone.0094530.g001

Methods

Ethics statement

All animal experiments were carried out according to the principles of the EU directive 86/609, NIH publication no. 85–23 (revised 1985), and the national animal experimentation act. The study was approved by the National Animal Experimentation Board under the Danish Government Ministry of Food Affairs according to EU directive 86/609, license number 2007/561−1434-C3.

Animals

NOD/BomTac mice were obtained from Taconic Europe A/S, Ry, Denmark and kept under barrier-protected conditions according to the FELASA guidelines [22]. The mice had free access to acidified drinking water and were fed standard Altromin 1324 diet (Altromin, Lage, Germany).

Reagents and antibodies

Crude wheat gluten and ovalbumin (OVA) were obtained from Sigma (Sigma, St. Louis, MO), while gliadin was from Fluka (Sigma). Phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma. The following monoclonal antibodies (mAbs) as well as isotype controls were purchased from BD Biosciences (BD Biosciences, Mountain View, CA): Alexa Fluor 488-conjugated rat anti-mouse IL-2 (JES6-5H4, IgG$_{2b}$), IL-4 (11B11, IgG$_1$), IFN-γ (XMG1.2, IgG$_1$), FITC-conjugated rat anti-mouse IL-10 (JES5-16E3, IgG$_{2b}$) and CD8 (53-6.7; IgG2a,κ), PerCP-Cy5.5-conjugated hamster anti-mouse CD3 (145-2C11; IgG1,κ), rat anti-mouse CD4 (RM4-5; IgG2a,κ), CD8 (53-6.7; IgG2a,κ) and PE-conjugated hamster anti-mouse γδ T cell receptor (GL3; IgG2,κ) mAbs. Mouse Treg staining kit Cat.No. 88–8111, PE-conjugated rat anti-mouse Foxp3 mAb (FJK-16s; IgG2a,κ) and FITC-conjugated rat anti-mouse CD4 mAb (RM4-5; IgG2a,κ) were from eBioscience (eBioscience, San Diego, CA). The anti-mouse CD4 mAb (BD Biosciences) was used

in combination with intracellular cytokine staining using Cytofix/Cytoperm kit (BD Biosciences), while the anti-mouse CD4 mAb (from the eBioscience kit no. 88–8111) was used when detecting Foxp3$^+$CD4$^+$ T cells (with no prior PMA inomycin stimulation) by using the Treg staining kit 88–8111.

Intranasal immunization and monitoring of diabetes

Non-anesthetized 4-week-old NOD female mice (n = 16 per group) were intranasally (i.n.) given 50 µg of OVA, gliadin, and/or gluten in a total volume of 10 µl (5 µl per nostril). Gliadin, gluten as well as OVA were dissolved in acidified (0.2% acetic acid) saline solution. Animals were immunized 5 times every other day. Five animals per group at the age of 13 weeks were used in separate experiments for insulitis scoring. NOD mice kept in our facilities start to progress to clinical onset of diabetes (>12 mmol) at the age of 14–15 weeks. Thus, at the age of 13 weeks these NOD mice have most of their islets affected by various stages of the mononuclear infiltrate, i.e. autoimmune aggression against β-cells. In order to test whether i.n. gliadin vaccination may prevent diabetes also in animals with advanced autoimmune reaction against β-cells, i.n. gliadin was tested in 13-week-old prediabetic NOD mice (n = 20 per group). For diabetes incidence studies, 16 to 20 mice per group were monitored for 210–240 days. NOD mice were inspected daily for diabetes and from 10 weeks of age screened weekly for glycemia with Glucometer FreeStyle mini (Hermedico, Brøndby, Denmark). Diagnosis of diabetes was based on two consecutive positive blood glucose readings >12 mmol/l during three days.

Histology and insulitis scoring

Insulitis scoring was performed on hematoxylin & eosin stained pancreata from non-diabetic NOD females (n = 5) at the age of 13 weeks; the age at which non of the animals progressed to clinical onset of diabetes in our SPF animal facility, while in control groups majority of islets were affected by some presence of mononuclear infiltrate (insulitis grades 1–4). One half of the pancreata was fixed
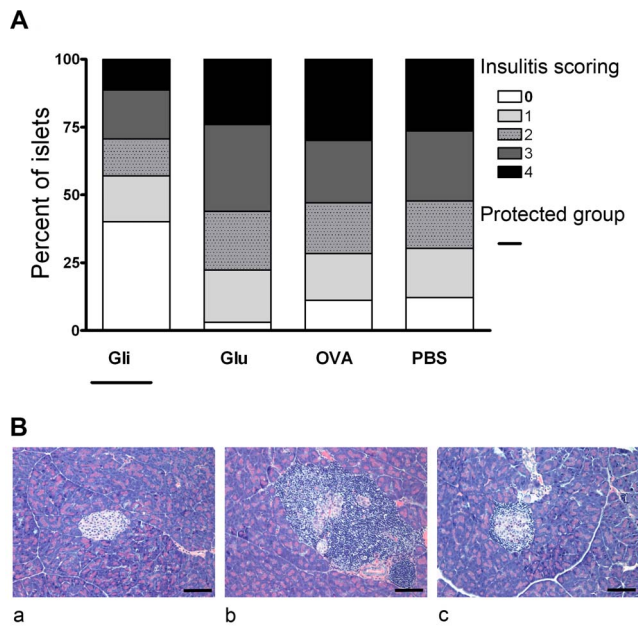
A

B

Figure 2. Intranasal administration of gliadin but not gluten prevented development of insulitis in NOD mice. (A) Insulitis was assessed by histological examination at 13 weeks of age before clinical onset of diabetes. The five grades (0–4) for insulitis scoring are described in the Research Design and Methods. Five mice per group and a minimum of 25 islets/mouse were scored and this experiment was carried out independently of diabetes incidence studies. (B) Photomicrographs of H&E stained histological specimens documenting an example islet of grade level 0 (a), 4 (b) and 3 (c) used for insulitis scoring. Example photomicrographs of grade level 0 (a), 4 (b) and 3 (c) are from 13-week-old NOD mice treated with gliadin, OVA, and gluten, respectively. Scale bars: 100 μm.
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in 4% formaldehyde, embedded in paraffin, cut in 5 μm sections and stained with hematoxylin & eosin for insulitis scoring. The grades for insulitis scoring were as follows: 0, normal islet; 1, intact islet with few scattered mononuclear cells in the surroundings; 2, peri-insulitis; 3, insulitis (<50% of the islet infiltrated); 4, severe insulitis (>50% of the islet infiltrated). A minimum of 25 islets were scored for each mouse and this experiment was carried out independently of diabetes incidence studies. Data are expressed as average percentage of islets affected by the 5 (0–4) insulitis grades (percent of islets) calculated from 5 animals per group.

Flow cytometry

Single cell suspensions of 8-week-old, normoglycaemic animals were prepared from the following mucosal lymphoid tissues: the nasal-associated lymphoid tissue (NALT), pancreatic lymph nodes (PLN), and mesenteric lymph nodes (MLN). Spleens (S) and systemic (inguinal) lymph nodes (ILN) were used as non-mucosal controls. For detection of Foxp3$^+$CD4$^+$ Tregs, intracellular staining for Foxp3 was carried out using the Treg staining kit 88–8111 (eBioscience) following the manufacturer procedure. When detecting intracellular cytokines, isolated cells were stimulated in vitro with a mixture of PMA (25 ng/ml) and ionomycin (1 μg/ml) in RPMI-1640, 10% FCS for 4 hours at 37°C, 5% CO_2 in the presence of Golgi Stop (Cytofix/Cytoperm kit, BD Biosciences) before subsequent staining for selected surface and intracellular markers. Titration experiments were performed to determine optimal lengths (1–6 hours) and concentrations of PMA/ionomycin stimulation. Unstimulated cells cultured in the presence of Golgi Stop were used as controls. For surface staining,

cells were incubated in FACS buffer with relevant mAbs for 30 minutes on ice. Fc block (CD16/CD32) was from BD Biosciences. For intracellular staining of cytokines, live cells were first stained for surface markers, then fixed/permeabilized with the Cytofix/Cytoperm kit following the manufacturer procedure. No difference in the staining was observed when comparing sequential versus one step procedure. Thus, anti-Foxp3 and an anti-cytokine mAbs were added in a single incubation step. Cells from OVA- and gliadin-treated mice were prepared, stained and measured on the same day. Cells were then analyzed by flow cytometry using a FACSscan (BD Biosciences), and data were analyzed by use of CellQuest (BD Biosciences), WinMDI 2.8 and/or FlowJo (TreeStar) software. Only very few cytokine-positive cells were detected in unstimulated controls. Isotype control antibodies were used to determine the amount of non-specific binding, and propidium iodide was used to localize and assess proportion of dead cells prior their fixation/permeabilization.

Statistics

The cumulative diabetes incidence was assessed using the Kaplan-Meier estimation and contingency tables. Log-rank test and Chi-squre test were used for comparisons between groups. Other results are expressed as mean ±SEM, and the level of significance (p<0.05) was assayed by two-sample analysis (unpaired t-test) or ANOVA followed by the Bonferroni multiple comparison test (comparison of multiple groups in insulitis scoring).

Results

I.n. administration of gliadin leads to reduced diabetes incidence in NOD mice

As shown in Fig. 1., five intranasal administrations of gliadin (50 μg) to 4-week-old NOD females significantly decreased the diabetes incidence to 56% in comparison to mice treated with OVA with a diabetes incidence of 100%, p = 0.001 (by log-rank test) and to PBS-treated controls (94%, p = 0.008). There were no substantial differences in the development of diabetes incidence between the phosphate-buffered saline (PBS) and OVA-treated control groups. I.n. administered wheat gluten consisting mainly of glutenins and gliadins and thus comprising the gliadin fraction, although in smaller amounts, had no effect on the diabetes incidence (81% at 210 days) compared to PBS and OVA controls (Fig. 1). The difference in diabetes incidence between the gluten (81%) and gliadin (56%) treated groups was again statistically significant at p = 0.029 (by log-rank test). Additionally, we investigated a more intense i.n. application scheme in which the five intranasal application of gliadin (50 μg) every other day were first applied to 4-week-old NOD mice and then repeated 2 times more, each time with a 10-day break interval. However, this high-frequency scheme of i.n. gliadin resulted in no significant diabetes prevention in NOD mice (data not shown) and is in accord with data from a mathematical model of NOD mice published by Fousteri G. et al. [23] in which high-frequency i.n. immunizations also failed in simulated disease protection. We also tested whether i.n. administration of gliadin leads to induction of tolerance but found no differences among the gliadin, OVA (and PBS) i.n. treated groups of mice in serum anti-gliadin IgG after s.c. immunization with gliadin in CFA as well as in cytokine recall responses (IFN-γ IL-5 and IL-10) after in vitro restimulation of MLNs suspensions (data not shown).

Reduction of insulitis by i.n. administration of gliadin

Insulitis scores from 13-week-old NOD female mice are overviewed in Fig. 2. Gliadin-treated NOD females (insulitis score
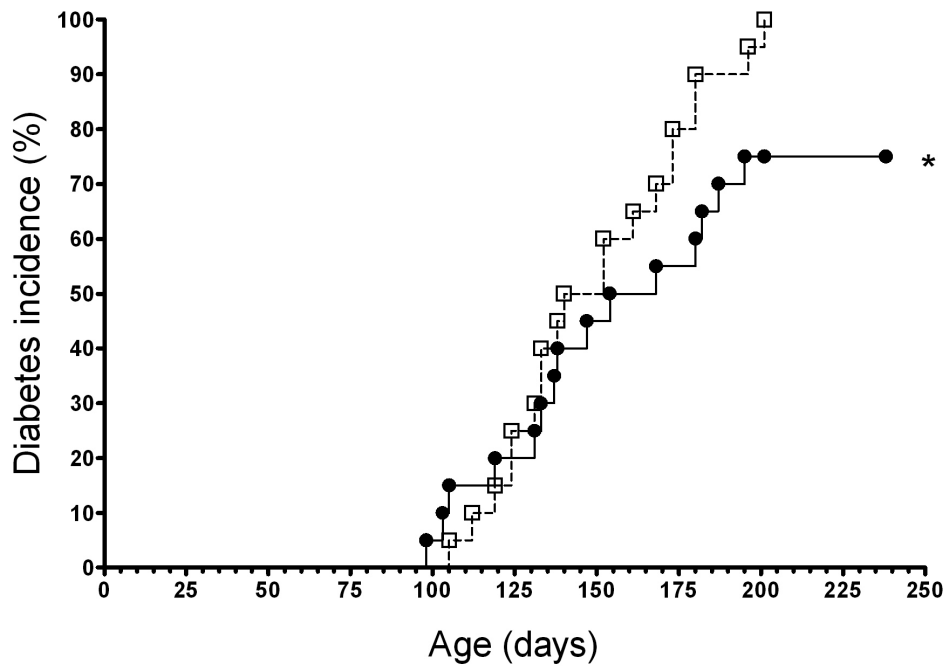
Figure 3. Intranasal administration of gliadin in prediabetic NOD mice with advanced insulitis decreases diabetes incidence. Groups (n = 20) of 13-week-old NOD female mice received five i.n. administrations (every other day). A statistically significant decrease in diabetes incidence was found at the age of 240 days in prediabetic NOD mice treated with gliadin (closed circle) compared to OVA control (opened square); *p = 0.017. While control OVA-treated mice display 100% diabetes incidence, only 75% gliadin-treated littermates progressed to the disease at 240 days.
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1.44±0.11) revealed statistically significant, less destructive insulitis compared to OVA (2.44±0.11, p<0.001) and PBS (2.36±0.10; p<0.001), but also to gluten (2.55±0.09; p<0.05) treated groups (Fig 2A). There were no significant differences among the gluten-treated and control (PBS, OVA) groups. Thus, in accord with the diabetes incidence data, insulitis scoring indicated a significant beneficial effect of gliadin on islet preservation. Animals used for insulitis scoring were from time different i.n. experiment. Fig. 2B illustrates examples of three different grades used for insulitis scoring.

I.n. gliadin reduces diabetes incidence even in 13-week-old NOD mice just before the clinical onset of diabetes

Five i.n. administrations of gliadin to prediabetic NOD females at the age of 13 weeks decreased the diabetes incidence compared to OVA-treated controls (p = 0.017, contingency tables and Chi-squre test, Fig. 3). Thus, i.n. administration of gliadin could rescue a small, but statistically still significant, proportion of the animals from progressing towards manifestation of the disease with glycemia values still below 12 mmol, in spite of a high degree of autoimmune infiltrate and damage present at this age in endocrine pancreata of NOD mice.

I.n. administration of gliadin increases number CD4+Foxp3+ T cells in mucosal but not in non-mucosal lymphoid organs

I.n. administration of gliadin to NOD mice led to increased number of CD4+Foxp3+ T cells at 8 weeks of age. Increased proportion of these was gliadin-specific in comparison with the i.n. administered control protein - OVA at the site of the antigen administration - in the NALT and in the mucosal draining lymph nodes of the pancreas – PLN, and MLN. Thus, an increase of CD3+CD4+Foxp3+ cells was found in NALT (p = 0.013), MLN

(p = 0.014) and PLN (p = 0.019) after i.n. gliadin vaccination (Fig. 4A). These data are significant also when expressed as proportion of CD4+ helper T cells (NALT, p = 0.019; MLN, p = 0.011; PLN, p = 0.049), (Fig. 4B and C). Interestingly, the i.n. gliadin-mediated increase of Foxp3+ T cells was not found in other non-mucosal lymphoid organs such as spleen and control systemic ILN (Fig. 4).

I.n. administration of gliadin leads to increased numbers of γδ T cells in mucosal but not in non-mucosal lymphoid organs

I.n. administration of gliadin to NOD mice led to a mucosal-specific accumulation of γδ T (CD3+) cells at the age of 8 weeks. Thus i.n. gliadin led to a substantially increased frequency of γδ T cells in NALT (p = 0.004), MLN (p = 0.004), and PLN (p = 0.001), but not in non-mucosal lymphoid organs such as spleen and ILN compared to OVA-treated controls (Fig. 5A and B). The increased frequency of γδ T (CD3-gated) cells was found both within the CD8- γδ T cell subset (NALT, p = 0.027; MLN, p = 0.013; PLN, p = 0.001) as well as in γδ T cells expressing CD8 marker (NALT, p = 0.002; MLN, p = 0.008; PLN, p = 0.007) (Fig. 5C and D). In gliadin-treated NOD mice, there was a shift towards increased proportion of CD8+ γδ T (CD3+) cells at the site of the immunization - the NALT and MLN. On the other hand, a relatively higher proportion of CD8- γδ T (CD3+) cells was detected in the draining lymph nodes of pancreas - the PLN (Fig. 5C and D).

Cytokine profiles of CD4+Foxp3+ and γδ T cells after i.n. administration of gliadin in mucosal and non-mucosal lymphoid organs

I.n. administration of gliadin is associated with increased amount of IL-10 and decreased amount of IL-2, IL-4 and IFN-γ in CD4+Foxp3+ T cells, preferentially in mucosal lymphoid organs in 8-week-old NOD mice. Following PMA/ionomycin stimulation
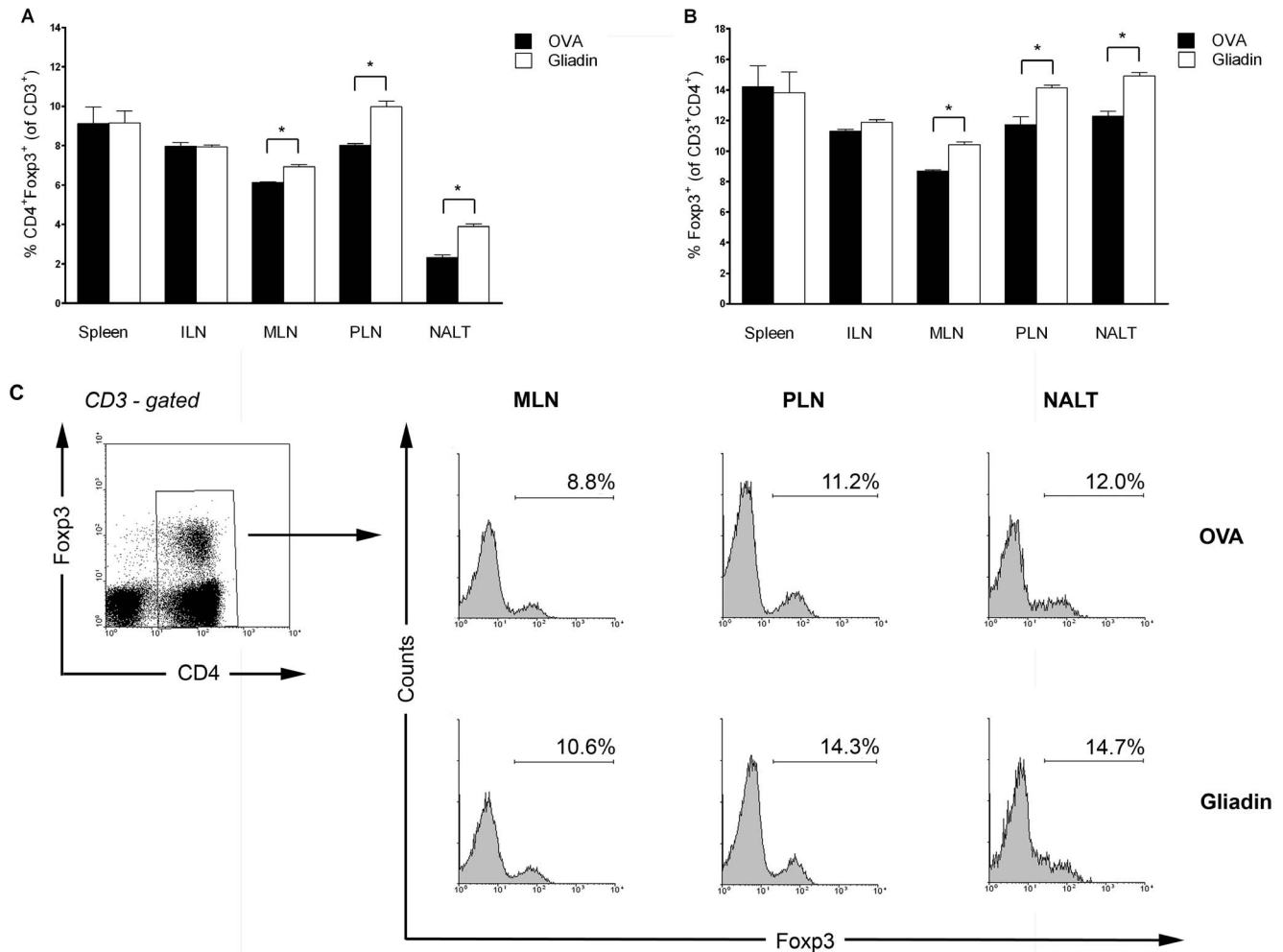
Figure 4. Induction of CD4$^+$Foxp3$^+$ T cells by intranasal administration of gliadin. (A) Proportion of CD4$^+$Foxp3$^+$ cells within T (CD3-gated) cells in mucosal (NALT, MLN, PLN) and non-mucosal compartments (Spleen, ILN) after i.n. gliadin (white bars) or OVA (black bars) vaccination in 8-week-old NOD female mice. (B) Proportion of CD4$^+$Foxp3$^+$ cells expressed as percentage of CD3$^+$CD4$^+$ T cell subset; lymphoid organs and bars as ad A. (C) Example FACS analysis of CD4$^+$Foxp3$^+$ T cells in MLN, PLN and NALT of gliadin and OVA-treated NOD mice. Cells were gated according to the CD3 parameter and Foxp3 expression analyzed within the CD4-positive cells (histograms). Individual measurements were performed on cells pooled from 2-3 experimental animals. Data are expressed as mean values ±SEM and represent an example of two independent experiments. *p<0.05.
doi:10.1371/journal.pone.0094530.g004

in vitro, an increased number of IL-10 positive CD4$^+$Foxp3$^+$ cells was found both in MLN (p<0.01) and PLN (p<0.05), but also in non-mucosal ILN (p<0.05) of the gliadin-treated NOD mice at the age of 8 weeks (Fig. 6B and C). On the other hand, CD4$^+$Foxp3$^+$ T cells from the gliadin-treated group displayed lower potential to produce IL-4 (MLN, p<0.001; NALT, p<0.05), IL-2 (ILN, p<0.01; NALT, p<0.05), and IFN-γ (MLN, p<0.05; PLN, p<0.05; NALT, p<0.05) (Fig. 6A, D and E). Only very few cytokine-positive cells were detected in unstimulated controls (example data in Fig. 6F). The most consistent difference was observed in the IFN-γ production of CD4$^+$Foxp3$^+$ T cells that was significantly reduced in all the mucosal lymphoid organs studied, including the pancreatic draining PLNs (Fig. 6E).

Within the CD4$^+$Foxp3$^-$ subset of T cells, only a small percentage of cells was positive for IL-4, IL-10 and IFN-γ. Substantially more CD4$^+$Foxp3$^-$ T helper cells were positive for IL-2 and a significantly increased number of IL-2 producing CD4$^+$Foxp3$^-$ T helper cells was detected in all organs studied (Fig. 6D). IL-10 positive CD4$^+$Foxp3$^-$ T cells were also more frequent in all studied organs except for NALT in the gliadin-

treated group (Fig. 6B and C). A decreased amount of IL-4 was found in all mucosal (MLN, p<0.05; PLN, p<0.01; NALT, p<0.05) lymphoid tissues of gliadin-treated mice, whereas IFN-γ was increased in spleen (p<0.01) and MLN (p<0.05), (Fig. 6A and E).

Almost no IL-2 and IL-4 positive γδ T cells were detected after in vitro restimulation with PMA and ionomycin (data not shown) regardless of the i.n. treatment. Although i.n. gliadin led to significantly increased induction of γδ T (CD3$^+$) cells in all studied mucosal lymphoid organs (Fig. 5), γδ T cells displayed no substantial differences in positivity for IL-10 as regards the i.n. treatment with gliadin and OVA (Fig. 7C). Diabetes-preventive i.n. administration of gliadin was associated with a decreased number of IFN-γ positive γδ T cells in MLN (p<0.05) and a similar tendency was observed in their PLN (Fig. 7A and B).

Discussion

We have shown that five intranasal administrations of 50 µg gliadin in 4-week-old NOD mice significantly reduce penetrance of diabetes as well as the level of insulitis. This gliadin treatment
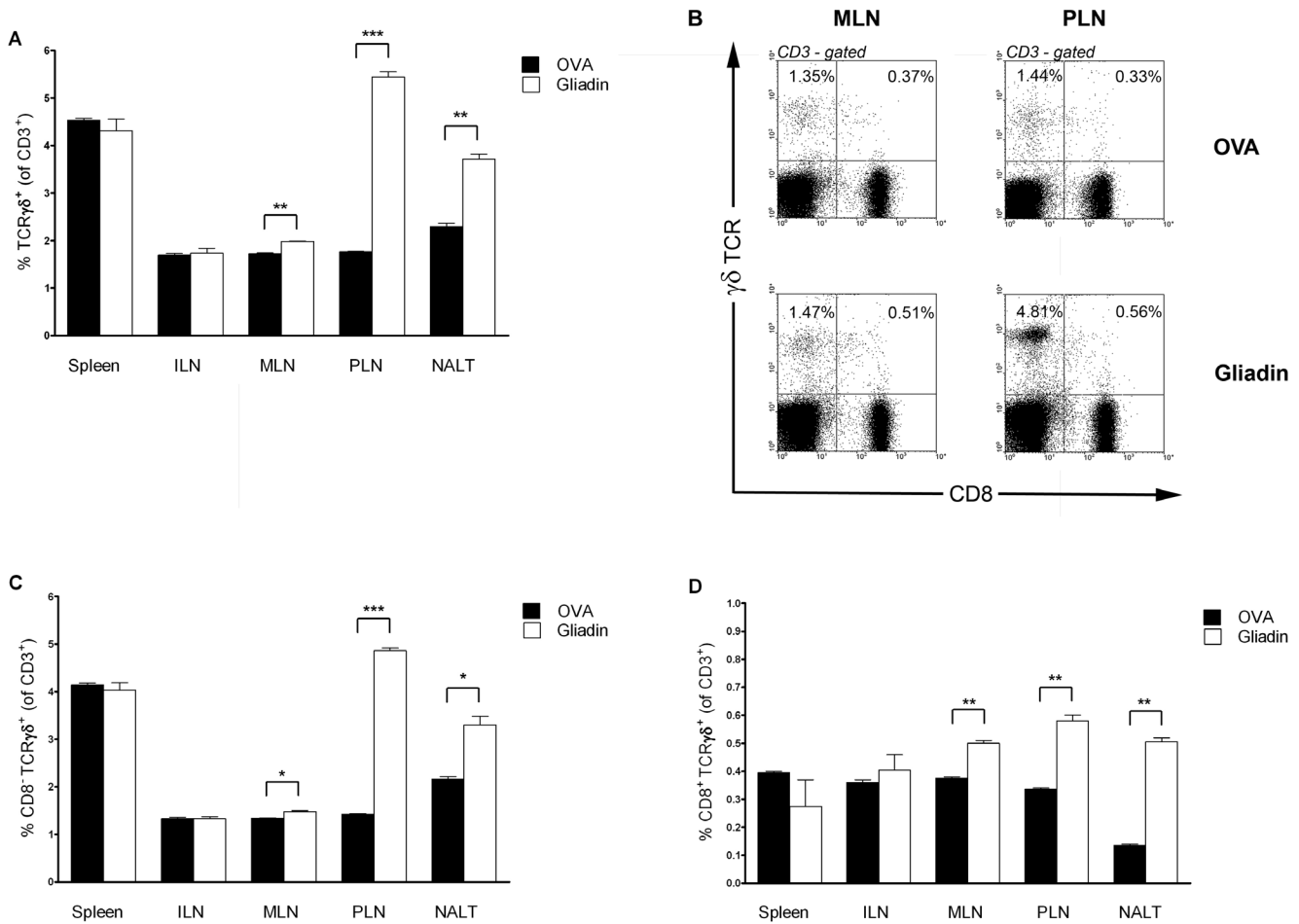
Figure 5. Effect of i.n. administration of gliadin on frequency of γδ T cells. (A) Proportion of γδ T cells within T (CD3-gated) cells in mucosal (NALT, MLN, PLN) and non-mucosal compartments (Spleen, ILN) after i.n. gliadin (white bars) or OVA (black bars) administration in 8-week-old NOD female mice. Panels C and D show further analysis of the γδ T cells according to their CD8 expression; lymphoid organs and bars as ad A. (C) Proportion of CD8⁻ γδ T cells expressed as percentage of CD3⁺ T cells. (D) Proportion of CD8⁺ γδ T cells expressed as percentage of CD3⁺ T cells. (B) Example FACS analysis of γδ T cells (CD3-gated) in MLN and PLN of NOD mice treated i.n. with gliadin or OVA. Individual measurements were performed on cells pooled from 2–3 experimental animals. Data are expressed as mean values ±SEM and represent an example of two independent experiments. *p<0.05, **p<0.01, ***p<0.001.
doi:10.1371/journal.pone.0094530.g005

was accompanied by an increase of CD4⁺Foxp3 T cells and much higher increase of γδ T cells in mucosal lymphoid compartments. Moreover, i.n. gliadin can even rescue a small fraction of prediabetic 13-week-old NOD mice (with a high degree of insulitis) from progressing to clinical onset of the disease. Thus, in this study an environmental antigen, closely related to the development of T1D, has been successfully applied in the disease prevention.

Prevention of T1D in NOD mice by i.n. gliadin was associated with an increased proportion of CD4⁺Foxp3⁺ and even more significant increase of γδ T cells specifically in the mucosal lymphoid compartments, but not in systemic lymphoid organs such as spleen and ILN (Fig. 4 and 5). Thus, these cells were found at the site of the i.n. application (NALT) at the draining lymph nodes (PLN) of the target organ, the pancreas, as well as in gut draining mucosal lymph nodes, MLN. This distribution pattern supports the concept of "common mucosal system" [24]. The increase of γδ T cells and Foxp3⁺ Tregs specifically in the pancreatic-draining PLN and also in MLN, in which priming of diabetogenic cells has been reported [25], points to possible mechanism of bystander suppression [26]. The increased propor-

tion of IL-10 in Foxp3⁺ Tregs, preferentially within the mucosal compartment, and a decrease of IFN-γ in both Foxp3⁺ Tregs and γδ T cells after i.n. gliadin (Fig. 6 and 7) are in accord with the previously reported role of IL-10 cytokine produced by disease protective Tregs [5,26,27].

It has been suggested that a deficiency of Tregs could be associated with T1D development, and defective suppressor function in CD4⁺CD25⁺ T cells was reported in T1D patients [28,29]. Although we found increased proportions of both Foxp3⁺ and γδ T cells after i.n. gliadin, the effect on γδ T cells seems to be substantially larger. γδ T cells are not generally considered as a typical Treg subset, however there are several lines of evidence for their involvement and even regulatory role in T1D [5,30–32]. We have documented that NOD mice display an increased proportion of γδ T cells at onset of diabetes [33]. γδ T cells specific for insulin peptide B:9–23 were also reported in NOD mice [34]. On the other hand, γδ T cells play an important role in induction and maintenance of oral tolerance [35]. It has been shown that in the neonatal thymectomy NOD model of T1D, gut intraepithelial CD8⁺γδ T cells can prevent development of diabetes, and proper development of intraepithelial γδ T cells is required for induction
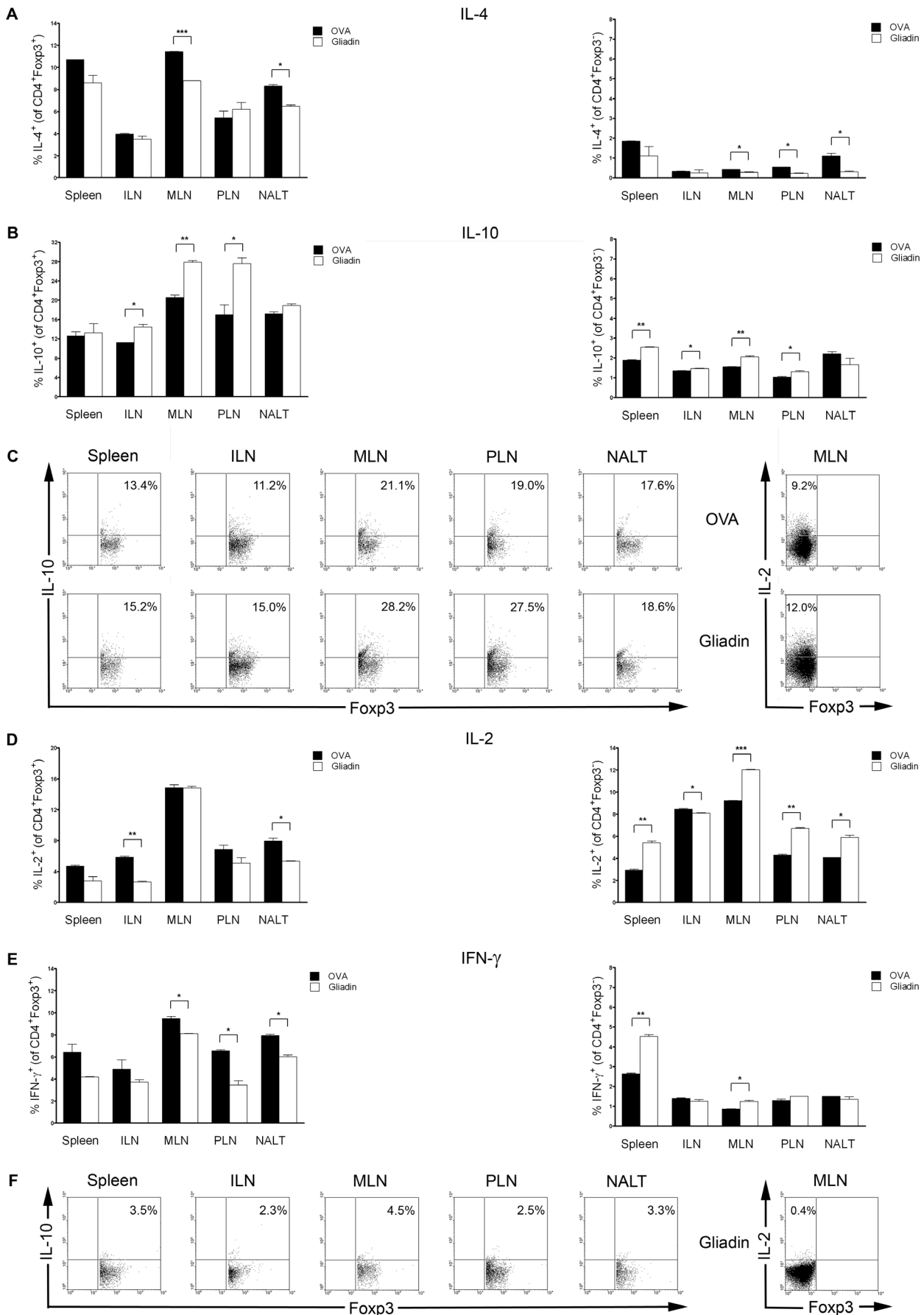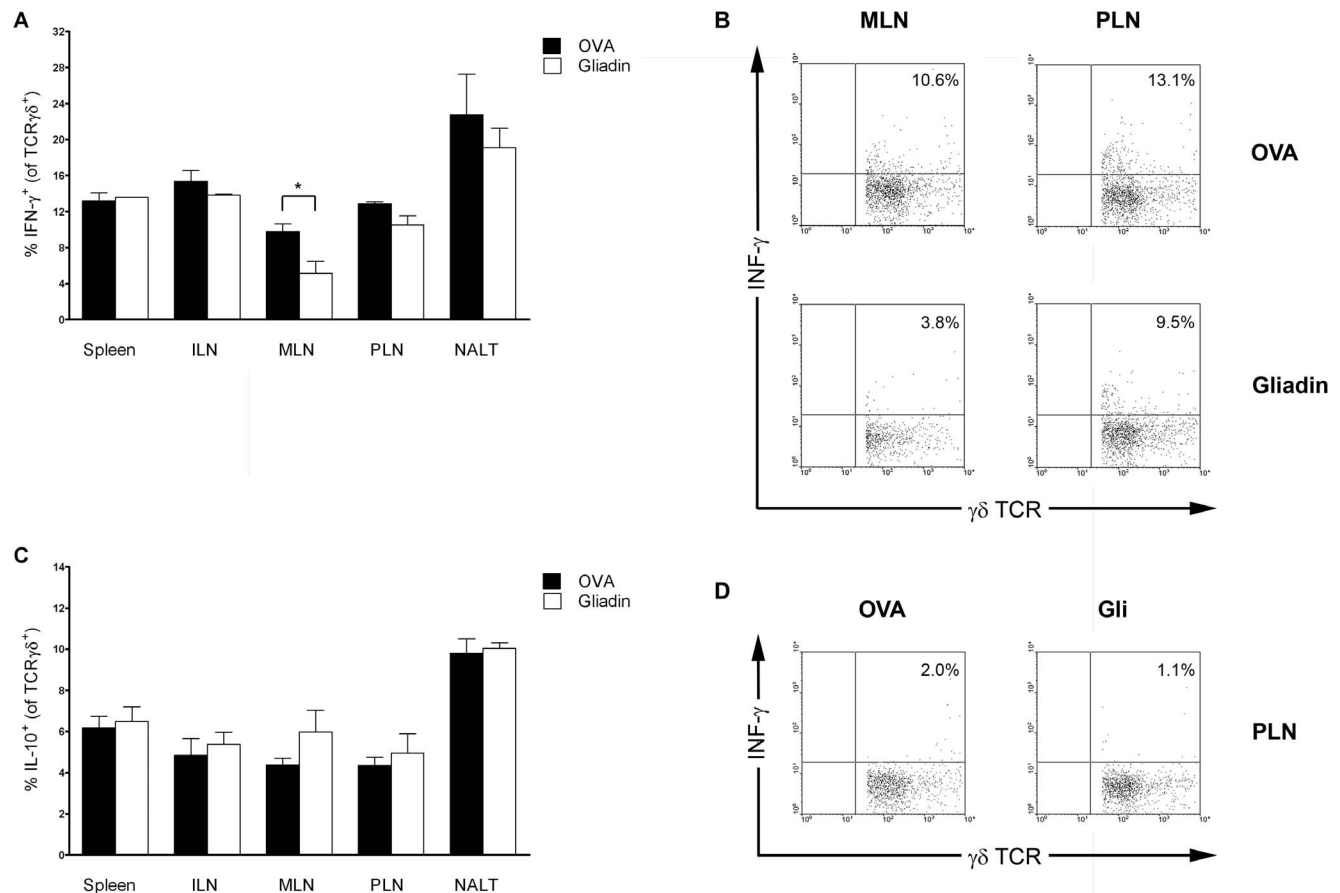
Figure 6. Cytokine profiles of CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ cells after i.n. administration of gliadin. Frequency of cytokine-positive cells in mucosal (NALT, MLN, PLN) and non-mucosal compartments (Spleen, ILN) after 4 hours unspecific in vitro restimulation with PMA/ionomycin is shown in panel A (IL-4), B (IL-10), D (IL-2) and E (IFN-γ). Left panels display cytokines expression in CD4⁺Foxp3⁺ and right panels in CD4⁺Foxp3⁻ cells. (C) Example FACS analysis of IL-10 expression within CD4⁺Foxp3⁺ (Spleen, ILN, MLN, PLN and NALT) and IL-2 positive cells within CD4⁺Foxp3⁻ subset (MLN) of 8-week-old NOD mice vaccinated i.n. with gliadin or OVA at 4 weeks of age. (F) Example of IL-10 and IL-2 positive cells in unstimulated controls (cell subsets and organs as at C). Individual measurements were performed on cells pooled from 2–3 experimental animals at age of 8 weeks. Data are expressed as mean values ±SEM, i.n. gliadin (white bars), i.n. OVA (black bars). *p<0.05, **p<0.01, ***p<0.001.
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of regulatory CD4⁺CD25⁺ T cells by oral insulin [32]. Interestingly, intranasal aerosol application of the whole insulin molecule in NOD mice led to induction of CD8⁺γδ T cells capable of preventing development of diabetes in an adoptive cotransfer model [5]. This study corresponds with our data documenting that i.n. administration of another whole-molecule antigen - gliadin - also led to preferential induction of γδ T cells.

Several of the animal-tested T1D autoantigens proceeded to human trials e.g. oral or intranasal insulin administration in humans at risk of type 1 diabetes (DPT-1, INIT). While some human trials are in progress, others such as the Diabetes Prevention Trial-1 (DPT-1) with oral or s.c. and i.v. insulin or s.c. GAD65/alum failed to show a protective effect [10,36–38]. Also in other autoimmune diseases, human trials with oral autoantigens have not led to satisfactory outcomes - (reviewed in [39].

Nevertheless, there are a few aspects that may question the use of β-cell autoantigens in mucosal prevention of autoimmune diseases, in particular T1D. Firstly, type 1 diabetes is an autoimmune disease for which the Witebsky and Rose's original autoimmune criterion of disease-induction with a specific autoantigen has never been met [40]. In fact, immunizations with neither β-cell autoantigens nor pancreatic extract together with adjuvants were able to induce T1D, reviewed in [41]. Secondly, since mucosally administered antigens can induce both tolerance as well as immunity, any use of "self" antigens requires extensive testing and cautions for induction of autoimmunity [9,42,43]. Thus, vaccinations with β-cell related autoantigens have not so far proven valuable for humans, although combination therapy with

Figure 7. Expression of IL-10 and IFN-γ in γδ T cells after i.n. administration of gliadin. Proportion of IFN-γ (panel A) and IL-10 (panel C) positive γδ T cells within mucosal (NALT, MLN, PLN) and non-mucosal compartments (Spleen, ILN) after i.n. gliadin (white bars) or OVA (black bars) administration in 8-week-old NOD female mice. (B) Example FACS analysis of IFN-γ expression within γδ T cells in MLN and PLN of NOD mice treated i.n. with gliadin or OVA after 4 hours in vitro restimulation with PMA/ionomycin. (C) Example of IFN-γ positivity within γδ T cells of unstimulated controls (PLN) from gliadin-treated NOD mice. Individual measurements were performed on cells pooled from 2–3 experimental animals. Data are expressed as mean values ±SEM, *p<0.05.
doi:10.1371/journal.pone.0094530.g007

immunosuppressive anti-CD3 mAb seems to be promising [11,26].

In contrast to gliadin, i.n. administration of gluten did not lead to prevention of diabetes in NOD mice. This could be due to a lower dose of gliadin present in the gluten fraction. We hypothesize, it might also be a matter of availability - e.g. transports on mucosal surfaces and presentation by DCs, as glutenins are formed by much higher molecular weight polypeptide chains that tend to form a molecular net, thus may limit availability of various gliadins within the gluten fraction. The effect of gliadin on immune responses is not fully understood. Prevention of T1D by i.n. gliadin did not induced specific tolerance to gliadin. This might be due to the fact that the 4- or even 13-week-old NOD mice fed standard gluten-containing diet have already well-established oral tolerance to gliadin, since a very short period of oral antigen exposure is needed for induction of oral tolerance [44]. Gliadin induces activation of innate immune mechanisms and maturation of dendritic cells [45]. While gliadin-induced inflammatory cytokine production was described as MyD88-dependent, TLR2 and TLR4 were reported as not involved in the gliadin-induced signaling pathway [46,47]. Furthermore, gliadin derived peptides may trigger T cell specific responses but also stimulate innate immunity response [48]. Gluten-free diets but also gluten content in diets influence diabetes incidence in animal models [15,16,49]. The etiological role of gliadin in T1D is supported by the study by Galipeau HJ et al. [50] documenting that while sensitization with gliadin induces only moderate enteropathy in humanized NOD-DQ8 mice, when combined with partial antibody depletion of Foxp3 Tregs it led to development of insulitis. The induction of mucosal γδ T cells by i.n. gliadin corresponds with our recent reciprocal finding: we found decreased proportions of γδ T cells in mucosal and non-mucosal compartments of BALB/c mice fed standard compared to a gluten-free diet [51].

The use of an external environmental substance, gliadin, in this study is novel and may have clinical implications. In addition, gliadin represents one of the most common food antigens in western countries, thus its safety in human application may be easier to test compared to β-cell related autoantigens [9,42,43].

In conclusion, our data show that i.n. mucosal application of gliadin is capable of significant prevention of diabetes and development of insulitis in NOD mice. In addition, i.n. gliadin displayed also small effect on preventing progression to clinical diabetes in just prediabetic animals with advanced autoimmune aggression within their islets. This prevention by an environmental antigen - gliadin - is associated with local, mucosal induction of γδ T cells and to much lesser extent CD4+Foxp3+ T cells. Because environmental factors play important roles in the recent increase of T1D, environmental antigens related to T1D should be considered for prevention trials. Our data suggest that intranasal, mucosal vaccination with gliadin may represent a novel and relatively safe approach to prevention and/or even early cure of type 1 diabetes.

Acknowledgments

Author Contributions

Conceived and designed the experiments: DPF PF KB. Performed the experiments: DPF PF. Analyzed the data: DPF PF KB AKH. Contributed reagents/materials/analysis tools: AKH KB DPF. Wrote the paper: DPF.

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3.2.2. Publication VIII.

Tlaskalová-Hogenová H, Stěpánková R, Kozáková H, Hudcovic T, Vannucci L, Tučková L, Rossmann P, Hrnčíř T, Kverka M, Zákostelská Z, Klimešová K, Přibylová J, Bártová J, Sanchez D, **Fundová P**, Borovská D, Srůtková D, Zídek Z, Schwarzer M, Drastich P, Funda DP. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. **Cell Mol Immunol., 2011; 8(2):110-20.** Review. IF (2015) = 5.193

This review addresses the importance of interplay of microbioms and mucosal barriers in shaping mucosal immune responses in postnatal development, and in development of inflammatory, autoimmune as well as neoplastic diseases. Use of germ-free and environmentally (microflora) defined, gnotobiotic animal models is accented in studying pathogenesis of these human diseases. More specifically, this review focuses on inflammatory bowel disease, celiac disease, type 1 diabetes, neurological and psychiatric diseases, rheumatic diseases, cardiovascular diseases and obesity, allergies and cancers.

With respect to this thesis, the review relates to second part of this thesis - experimental studies on the role of mucosal immunity and environmental factors in type 1 diabetes. The obvious role of environmental factors, the role of intestinal microflora and germ-free conditions, role of dietary factors (gluten) as well as gut permeability in development and prevention of type 1 diabetes are discussed in this review.

REVIEW

The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases

Helena Tlaskalová-Hogenová[1], Renata Štěpánková[1], Hana Kozáková[1], Tomáš Hudcovic[1], Luca Vannucci[1], Ludmila Tučková[1], Pavel Rossmann[1], Tomáš Hrnčíř[1], Miloslav Kverka[1], Zuzana Zákostelská[1], Klára Klimešová[1], Jaroslava Přibylová[1], Jiřina Bártová[2], Daniel Sanchez[1], Petra Fundová[1,3], Dana Borovská[1], Dagmar Šrůtková[1], Zdeněk Zídek[4], Martin Schwarzer[1], Pavel Drastich[5] and David P Funda[1]

Metagenomic approaches are currently being used to decipher the genome of the microbiota (microbiome), and, in parallel, functional studies are being performed to analyze the effects of the microbiota on the host. Gnotobiological methods are an indispensable tool for studying the consequences of bacterial colonization. Animals used as models of human diseases can be maintained in sterile conditions (isolators used for germ-free rearing) and specifically colonized with defined microbes (including non-cultivable commensal bacteria). The effects of the germ-free state or the effects of colonization on disease initiation and maintenance can be observed in these models. Using this approach we demonstrated direct involvement of components of the microbiota in chronic intestinal inflammation and development of colonic neoplasia (i.e., using models of human inflammatory bowel disease and colorectal carcinoma). In contrast, a protective effect of microbiota colonization was demonstrated for the development of autoimmune diabetes in non-obese diabetic (NOD) mice. Interestingly, the development of atherosclerosis in germ-free apolipoprotein E (ApoE)-deficient mice fed by a standard low-cholesterol diet is accelerated compared with conventionally reared animals. Mucosal induction of tolerance to allergen Bet v1 was not influenced by the presence or absence of microbiota. Identification of components of the microbiota and elucidation of the molecular mechanisms of their action in inducing pathological changes or exerting beneficial, disease-protective activities could aid in our ability to influence the composition of the microbiota and to find bacterial strains and components (e.g., probiotics and prebiotics) whose administration may aid in disease prevention and treatment.
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Keywords: allergy; hygiene hypothesis; intestinal permeability; leaky gut; probiotics

INTRODUCTION

The majority of epithelial surfaces of our body, such as the skin and mucosa, are colonized by a vast number of microorganisms; these represent the so-called normal microflora, the microbiota. The microbiota comprises mainly bacteria; however, viruses, fungi and protozoans are also present. Our microbiota contains trillions of bacterial cells, 10 times more cells than the number of cells constituting the human body. Most of the commensal bacteria are symbiotic; however, after translocation through the mucosa or under specific conditions, such as immunodeficiency, commensal bacteria could cause pathology. Bacteria are present at anatomical locations that provide suitable conditions for their growth and proliferation. Skin is predominantly colonized by bacteria in the skin folds. The upper airways, particularly the nasopharynx, harbor bacteria, as do some mucosal surfaces of the genital tract, although the greatest number of bacterial cells is found in the digestive tract. The oral cavity (tongue, teeth and periodontal tissues) harbors high numbers of bacteria (10^{12}). The stomach has only 10^3–10^4 bacteria, the jejunum harbors 10^5–10^6 bacteria and the terminal ileum harbors 10^8–10^9. However, the largest number of bacterial cells is found in the large intestine (10^{11} per gram

of intestinal content). A considerable portion, about 70%, of this microbial cosmos inside our body is composed of bacteria that cannot be cultivated by current microbiological methods. Microbial groups have been found to develop in close parallel with the human body and to depend on the physiological environment in unity with their hosts; hence, like most other higher organisms, humans are, in fact, supraorganisms. Our microbiota represents a complex ecosystem with enormous microbial diversity.[1,2] Molecular biological methods have allowed for a revolutionary advance in microbiological research: using these approaches, microbiological laboratories worldwide have begun to analyze the components of the human microbiota and to collaborate intensively in deciphering the human microbiome. It is noteworthy that the number of genes of our colonic microbiota exceeds the number of genes in the human genome by 150 times.[3] There are more than 50 bacterial phyla on Earth, but human gut-associated microbiota are dominated by four main phyla: *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*.[2] Fundamental comparative studies of human fecal microbiota have revealed the astonishing fact that each of us has a unique microbiota (i.e., there are considerable differences between the compositions of the microbiota of individuals). It has also been shown that the main bacterial populations comprising our microbiota stabilize during the first years of life. During this time, the microbiota develops and subsequently remains stable throughout our life in terms of the major bacterial populations, even after antibiotic treatments.[4] This large microbiome could produce an enormous quantity of molecules able to interact with the host; however, the role of these molecules remains to be elucidated. The existence of bacteria in the large intestine and their fundamental functions in nutrition and metabolism (fermentation of nondegradable oligosaccharides, metabolism of xenobiotics and activation or destruction of mutagenic metabolites) make the colonic microbiota a large fermentative organ.[5]

Metagenomic approaches have recently been used to demonstrate that the main functions of the small intestine microbiota may differ from the function of the colonic microbiota. The microbiota present in the small intestine is enriched in pathways and functions related to carbohydrate uptake and metabolism.[6] The small intestine contains the majority of immune cells in the body and is substantially involved in an appropriately functioning immune system; the small intestinal microbiota thus could play a more important role in development and maintenance of mucosal and systemic homeostasis. Dietary interventions and the administration of probiotics could be effective means of changing the composition of the relatively simple microbial community present in the small intestine and could thereby substantially affect this community's metabolic and immunomodulatory functions.

Although molecular biological analysis of the microbiota is providing new knowledge daily, functional studies concentrating on intensive analysis of the effects of the microbiota on the macroorganism are proceeding in parallel. The use of gnotobiological methods on experimental animals are an indispensable methodological tool in the study of the biological importance of the microbiota and the consequences of bacterial colonization. In these methods, mice or other species are bred by a complex technology in a sterile environment (i.e., free of live bacteria) in isolators and can then be colonized in a controlled way with defined strains of bacteria (including nonculturable species), and the effects of this colonization can be followed on both genetic and protein levels.[7,8]

MUCOSAL BARRIER FUNCTION

Epithelial surfaces have evolved protective mechanisms to resist microorganism invasion. Both mucosa and skin mediate contact between the organism and its external environment and there the organism encounters many antigenic, mitogenic and toxic stimuli present in food, normal microbiota and air. Moreover, most 'exogenous' pathogenic infections enter their host by the mucosal route. The mucosa and the internal environment of the organism are protected by the effective innate and adaptive immune systems. Almost 80% of the immunologically active cells of the body belong to the mucosal-associated immune system. The majority of these cells are present in tissues of the gastrointestinal tract, where the prevalence of immunogenic agents, including food and components of the microbiota, is the highest. Under physiological conditions, the gut is covered by the largest epithelial surface in the body (around 200 m^2 in humans), and it contains complex and poorly understood cell interactions that regulate responses to food antigens and to antigens of the normal bacterial flora.[9–15]

The barrier function of mucosal surfaces, particularly those of the intestine, is ensured by complex mechanisms acting on several levels. The microbiota itself forms an integral part of the natural mechanisms of mucosal surfaces and skin that safeguard the organism against pathogenic microorganisms. When the microbiota has an optimal composition, it prevents attachment and multiplication of pathogenic or virulent microorganisms on these surfaces and the invasion of these microorganisms into epithelial cells and the circulation. The intestinal microbiota plays an important role in pathogen resistance, both by direct interaction with pathogenic bacteria and by influencing the immune system.[16,17]

Mucins (highly glycosylated macromolecules) form the first barrier between the gut contents and epithelial cells, protecting them from direct contact with commensal bacteria and their components. Changes in the amount and/or the composition of mucus may lead to inflammatory responses.[18] The epithelial layer, which is covered by glycocalyx, forms a major barrier between the host and the environment. The epithelial layer of most mucosal surfaces consists of a single layer of interconnected, polarized epithelial cells. The epithelial layer of the gut mucosa is reinforced by junctions (tight junctions, adherens junctions and desmosomes) in the paracellular spaces between epithelial cells and forms an interconnected network. Tight junctions have been shown to act as a dynamic and strictly regulated port of entry that open and close in response to various signals such as cytokines and bacterial components, originating in the lumen, lamina propria and epithelium. Tight junctions participate in preserving cellular polarity and are regarded as key elements of intestinal diffusion mechanisms. The molecules forming tight junctions are connected to the cytoskeleton of epithelial cells and thus participate in determining the shape and structure of epithelial cells.[19] Epithelial cells participating in mucosal barrier function are conventional enterocytes (colonocytes in colon); goblet cells producing both mucus and trefoil peptides required for epithelial growth and repair; enteroendocrine cells producing neuroendocrine molecules having a paracrine effect; and Paneth cells secreting the antimicrobial peptides defensins. Neuropeptides, the products of the nervous system, are capable of increasing the permeability of tight junctions to macromolecules, thus modifying mucosal barrier function.[20]

The participation of innate immune factors in mucosal barrier function during the interaction with commensal microorganisms is now beginning to be appreciated. In addition to the well-known humoral components of innate immunity such as complement, lysozyme, lactoferrin and mannan-binding protein, other recently described factors have been intensively studied. An important humoral component of these non-specific innate mechanisms are the antimicrobial

peptides called defensins, widely distributed throughout the plant and animal kingdoms. Multiple types of these peptides are produced by Paneth cells, specialized cells present in the crypts of gut mucosa, and by other epithelial cells. In general, innate immune mechanisms are affected mainly by phagocytic cells (macrophages, neutrophils and dendritic cells) that can produce cytokines essential for inflammatory reactions and factors critical for the subsequent initiation of adaptive immunity. These cells initiate innate immune responses to microbes *via* the sensors called pattern recognition receptors.[21] These sensing structures, the Toll-like receptors, C-type lectin receptors, RIG-I-like receptors and nucleotide-binding domain and leucine-rich repeat containing proteins, sense pathogen motifs and transmit activation signals to their target cells. In addition to their strategic localization and absorptive, digestive and secretory functions, intestinal epithelial cells are equipped with various receptors to enable their participation in immunological processes. To prevent uncontrolled inflammatory responses to components of commensal microorganisms, the signaling pathways of these cells are tightly regulated by multiple molecules and pathways to ensure negative feedback mechanisms, similar to other mucosal innate immune cells.[16,22,23] We have previously shown that expression of the NOD2 molecule in the gut mucosa is affected by the presence of microbiota and that NOD2 expression influences the microbiota as well. The host *via* NOD2 and the intestinal commensal bacterial flora thus maintain homeostasis by regulating each other through feedback mechanisms.[24]

The main cells that present antigen to the adaptive arm of the mucosal immune system are dendritic cells.[25] Induction of tolerance or stimulation of a mucosal immune response depends on the participation of different populations of dendritic cells responsible for the activation of regulatory T-cell subpopulations.[26] Production of IL-10 and transforming growth factor-beta leads to the activation of regulatory T cells that inhibit the immune response and induce mucosal tolerance.[27] Pathogenic microorganisms induce the maturation of dendritic cells that lead to the activation of effector T cells indispensable for clearing infections and for the prevention of subsequent infection with the same or related pathogens.[25]

One of the main humoral defense mechanisms ensuring the barrier function of mucosal surfaces produced by the adaptive arm of the mucosal immune system is secretory immunoglobulin A (IgA). Polymeric secretory IgA is more resistant to proteolysis than other antibodies. Its primary task is to prevent both the adherence of bacteria to mucosal surfaces and the penetration of antigens into the internal environment of the organism. This is achieved by specific or nonspecific (using a reaction resembling lectin binding) mechanisms.[28–30] In individuals with selective IgA deficiency, the mucosal barrier is insufficient and is more permeable to allergens and other immunogens. Secretory IgA can react with some bactericidal substances contained in mucosal secretions (lactoperoxidase and lactoferrin) and transport them to bacterial surfaces.[28]

Mucosally administered antigens induce an immune response that is detectable not only locally, but also in circulation and in remote mucosal surfaces and exocrine glands.[31] Cells originating from organized mucosal lymphoid tissue migrate through lymph and blood after activation and home to mucosal surfaces and exocrine glands.[10,11,30] An example of the effect of migration and selective colonization by cells from the intestinal mucosal surfaces is the composition of the secretion from mammary glands: mother's milk. Apart from the nutritive components, mother's milk contains a number of immunologically nonspecific and specific factors and a large quantity of cells. These components protect the not yet completely developed intestine of the infant against infectious agents. The mammary gland is colonized by immune cells from the intestine of the enteromammary axis.[32] As a consequence of this colonization, the mammary secretions contain IgA antibodies and cells directed against antigens present in the maternal intestine, protecting the breastfed infant from threats present in its environment. This may involve bacteria from the maternal microbiota that colonize the intestine of the infant within the first 3 days of life and may have a pathological impact on the incompletely matured mucosa, as occurs in the case of necrotic enterocolitis. Therefore, the local protection provided for the infant's intestine by a number of molecules with immunomodulatory properties present in colostrum and milk, as well as maternal secretory antibody, is of major importance.[33]

THE ROLE OF THE MICROBIOTA IN POSTNATAL DEVELOPMENT OF INNATE AND ADAPTIVE IMMUNITY AND THE MUCOSAL BARRIER

The close symbiosis of the microbiota and human or animal hosts is the result of long evolution and mutual adaptation of both partners, which defines our ability to adapt to the ambient environment and defend ourselves against threats. The period in which the human host is most acutely influenced by the microbiota is the postnatal period, during which the germ-free neonate moves from the sterile environment of its mother's uterus into a world full of microorganisms and during which the neonate's mucosal and skin surfaces become gradually colonized. The composition of main bacterial populations does not stabilize until after the first few years of life. In this period, the microbiota gradually colonize the mucosal and skin surfaces of the neonate and exert the greatest effect on the development of the immune system.[34] The mode of neonate delivery is particularly important because infants delivered by caesarean section lack the first input of maternal bacteria, and their intestinal microbiota differ substantially.

Components of the intestinal microbiota play a crucial role in the postnatal development of the immune system. During the early postnatal period, the intestinal microbiota stimulates the development of both local and systemic immunity, while later on these components evoke inhibitory regulatory mechanisms intended to keep both mucosal and systemic immunity in check.[35–37]

The importance of the microbiota in the structural and functional features of the developing immune system was predicted by Professor Jaroslav Šterzl, who established the Laboratory of Gnotobiology at the Institute of Microbiology more than 50 years ago. This crucial development provided tools to study basic questions about the host–microbiota interaction using various animal models.[38–44] We have shown that microbial colonization of animals living in germ-free conditions results in an increase in immunoglobulin levels, the production of specific antibodies, substantial changes in mucosal-associated lymphocyte tissues and cell populations, changes in migration patterns and increases in the systemic immunological capacity.[35,40,42,43] In the early postnatal period, components of the normal microbiota induce a transient physiological inflammatory response in the gut associated with enlargement of the mucosal-associated lymphatic tissue and increases in its cellularity.[39,45]

The effect of microbial colonization on innate immune cells has been documented in our studies on the development of phagocytes, dendritic cells and intestinal epithelial cells.[24,46] Interestingly, the T-cell receptor repertoire is also influenced by colonization with microorganisms.[47] Recently we have studied the effect of the microbiota on the development of lymphatic subpopulations in BALB/c mice bred in

germ-free isolators or under conventional conditions and fed with sterile diets differing in contamination with microbial components. This study of lymphocyte subpopulations showed the mesenteric lymph nodes and Peyer's patches of germ-free mice fed by diets with lower lipopolysaccharide content contained fewer CD4^{+} T lymphocytes than did secondary lymphoid organs from mice housed under conventional conditions. Germ-free mice kept on a diet with a high content of nonliving microbial components had more CD4^{+} lymphocytes than animals kept on a diet with a low content of bacterial components. An important finding was that the development of regulatory (CD4^{+} FoxP3^{+}) T lymphocytes depends on the presence of the microbiota and bacterial components in the diet: germ-free mice on a diet containing small amounts of lipopolysaccharide had fewer regulatory T lymphocytes.[45]

Interestingly, the microbial colonization of germ-free mice also speeds up the biochemical maturation of enterocytes, resulting in a shift in the specific activities of brush-border enzymes nearly to the extent found in conventional mice.[48] Moreover, a similar introduction of microorganisms alters the synthesis of sugar chains in membrane-associated glycoproteins, which could influence the gut barrier function.[14,49,50]

PARTICIPATION OF COMMENSAL BACTERIA AND THEIR COMPONENTS IN THE DEVELOPMENT OF INFLAMMATORY, AUTOIMMUNE AND NEOPLASTIC DISEASES

While the major cause of death in the less developed world remains infectious disease, the major killers in the developed world are cardiovascular diseases and cancer. Moreover, the steadily increasing prevalence of chronic disorders, like allergy, arthritic diseases and other inflammatory and autoimmune diseases, is causing significant morbidity.[51,52] These disorders represent an important medical problem because they have a devastating impact on quality of life and require long-standing medical care.

The main characteristics of inflammatory and autoimmune diseases are tissue destruction and functional impairment caused by immunologically mediated mechanisms that are principally the same as those that function against pathogenic infections. Both living bacteria and their components and metabolites are clearly responsible for many of those immunomodulatory mechanisms.[53] Considerable work on autoimmune, inflammatory and neoplastic diseases is aimed at investigating the pathogenic role of environmental agents, including these microbial components.[54,55]

In some cases, impaired function of the intestinal barrier leads to an increase in antibodies directed against antigens present in the intestinal lumen. It was recently shown that the appearance of these antibodies or/and autoantibodies in individuals lacking clinical symptoms may have important predictive value for the development of inflammatory and autoimmune diseases.[56,57]

In the case of autoimmune diseases, considerable effort has been made to understand mechanisms leading to the loss of self-tolerance. Infections have often been considered to initiate the process in genetically predisposed individuals. One major hypothesis explaining how infectious components can cause autoimmune reactions is based on the concept of crossreactivity, also known as ''molecular mimicry'', the similarity between the epitopes of autoantigens and epitopes of harmless environmental antigens.[58,59] Infections may also trigger the development of autoimmunity through the inadequate activation of innate immune cells.[60] The adjuvant activity of microbial components may participate in the stimulation of antigen presenting cells such as dendritic cells that leads to the abnormal processing and presentation of self-antigens. Superantigens are microbial components that have been shown to be particularly effective in inducing inflammatory reactions.

Genome-wide association studies on large human cohorts are used to identify the role of genes mutated in chronic human diseases. These studies allow us to suggest not only the mechanisms but also the interacting environmental factors or infectious components involved in disease initiation and maintenance.[61,62]

Homeostasis of the intestinal mucosa may be disturbed by pathogenic microorganisms and toxins attacking the intestine or by inadequately functioning components of the immune system, as observed in immunodeficiency or in cases of dysregulated mechanisms of the mucosal immune system. The intestinal mucosa can be affected as a consequence of either insufficient activity or exaggerated activation of the immune system.[27,63] Various complex diseases may occur as a consequence of disturbances of mucosal barrier function or of changes in mechanisms regulating mucosal immunity to food or components of the microbiota.[64,65] Studies showing both interindividual differences and a disease-specific pattern in the composition of the microbiota in humans are of particular interest. Nevertheless, the complexity and interindividual variation of the gut microbiota composition in humans represents a confounding factor in the efforts to determine the possible significance of individual commensal microbial organisms in disease pathogenesis.

Patients often come to the clinic only after their disease has become symptomatic, making the understanding of the early events leading to disease difficult. Experimentally induced and spontaneously developing animal models of human diseases allow us to examine the role of genetic and environmental factors in early events during disease development, to elucidate the pathogenic mechanisms and to develop new preventive and therapeutic strategies, despite these models sometimes being too artificial to be comparable with human disease. Examples of diseases in which barrier dysfunction and involvement of the microbiota in human disease have been suggested and cases in which the use of germ-free or gnotobiotic animal models of disease were beneficial are listed below.

INFLAMMATORY BOWEL DISEASE (IBD)

Idiopathic IBD, Crohn's disease and ulcerative colitis are severe chronic disorders affecting approximately 0.2% of the human population. Despite intense study, the etiology and pathogenesis of these diseases remain unclear. The pathogenesis of IBD involves interactions among immune, environmental and genetic factors; the combination of these factors results in the induction of inflammation, subsequent development of mucosal lesions and repair. Disruption of T lymphocyte regulatory functions and impairment of the mucosal immune response to normal bacterial flora play a crucial role in the pathogenesis of chronic intestinal inflammation. This may implicate the loss of local physiological regulatory mechanisms and perhaps a breakdown of oral tolerance to luminal antigens in these diseases.[66–70] This suggests that the intestinal mucosa is one of the most sensitive indicators of immune dysfunction. The demonstration of abnormal T-cell responsiveness against indigenous microbiota in human IBD suggested that commensals may initiate and/or perpetuate the intestinal inflammation seen in IBD.[71] Recent results of genome-wide association studies performed in large cohorts of patients confirmed the previously suggested participation of microbial components in the development of Crohn's disease and ulcerative colitis.[72] Many of the mutations found were in genes encoding recognition, processing and killing of microorganisms and the regulation of immune processes.

Interestingly, some of these gene defects were also found in patients with other autoimmune diseases.

Several animal models of spontaneously developing intestinal inflammation suggest that innate immunity, mucosal barrier defects or disruption of T lymphocyte regulatory functions could lead to chronic intestinal inflammation. A number of genetically manipulated mice, such as mice deficient in IL-2 or IL-10, develop spontaneous chronic intestinal inflammation.[73] Interestingly, the disease can be prevented when these mice are reared in germ-free conditions.[74,75] Similarly, BALB/c mice develop a much milder form of acute dextran sulfate sodium induced colitis in germ-free conditions compared to conventionally reared mice.[76]

In our studies, we have used a T-cell transfer model of chronic colitis in germ-free and other gnotobiotic mice to elucidate the effects of colonization with defined mixtures of microbes on the development of intestinal inflammation. We observed that after the transfer of CD4$^+$CD45RB^{high} T cells into severe combined immunodeficient mice, severe inflammation was present in mice colonized with a cocktail of specific pathogen-free microbiota along with segmented, filamentous bacteria. Interestingly, germ-free mice, mice treated with segmented, filamentous bacteria alone or mice treated with the specific pathogen-free cocktail did not exhibit markers of severe intestinal inflammation.[77]

Oral treatment with lysed bacteria may influence the development of experimentally induced intestinal inflammation. We have shown that the severity of dextran sulfate sodium-induced intestinal inflammation in BALB/c mice is reduced by oral administration of a sonicated microbiota containing anaerobic bacteria.[78] Furthermore, we found that this effect could be modulated via the manipulation of the gut microbiota and immunomodulation of the mucosal and the systemic immune response.[79] Thus, the mechanisms of this protective and therapeutic effect should be elucidated more precisely, and this novel approach may be used for the development of a potential vaccine.

CELIAC DISEASE

Celiac disease is a chronic immune-mediated enteropathy that is triggered by dietary wheat gluten or related prolamins in genetically susceptible individuals. It is characterized by an increase in the cellularity (intraepithelial lymphocytes) and atrophy of jejunal mucosa. The autoimmune nature of this disease was confirmed by the presence of autoimmune mechanisms directed against several autoantigens, including the most diagnostically important autoantigen, tissue transglutaminase. The frequent association between celiac disease and other autoimmune diseases, particularly type 1 diabetes (T1D) and thyroiditis, suggests that celiac enteropathy shares certain pathogenic mechanisms with other autoimmune diseases.[80] Indeed, gut mucosal barrier dysfunction was repeatedly demonstrated and confirmed by genetic studies in patients with celiac disease and T1D.[81–83] Several intestinal viral triggers including adenovirus, hepatitis C virus, and rotavirus and bacterial infections capable of initiating or augmenting gut mucosal responses to gluten were suggested to play a role in the pathogenic mechanism of this disease.[84] Abnormal components found among the microbial inhabitants adhering to the diseased jejunal mucosa have been described and recently analyzed using new microbiological methods by Ou et al.[85] Profound changes in the fecal and duodenal microbiota composition of patients with active disease who are on a gluten-free diet have also been demonstrated.[86] Interestingly, some commensal bacteria, such as Escherichia coli, promoted the activation of innate immune cells by gliadin, whereas others (Bifidobacteria) exerted inhibitory effects.[87]

There are a limited number of suitable animal models for this disease. Using long-term intragastric application of gluten to Wistar-AVN rats starting at birth, we were able to induce the main features of gluten enteropathy: an increase in intraepithelial lymphocytes, crypt hyperplasia and shortening of the villi in the jejunal mucosa. Moreover, we found similar changes in mucosal structures after transfer of intestinal lymphocytes into the intestinal loops of inbred germ-free recipients. Changes appearing after gluten, but not albumin, feeding were inducible in germ-free rats, i.e., in the absence of microbiota, suggesting the activation of intestinal immune cells by this unique food protein.[88]

T1D

Type 1 (insulin-dependent) diabetes mellitus is one of the most well-studied organ-specific autoimmune diseases. It develops as a consequence of selective destruction of pancreatic insulin-producing beta cells within the islets of Langerhans. Autoimmune reactions against beta cells may arise from activation of the immune system in genetically predisposed individuals by environmental factors that bear epitopes similar to those expressed by the beta cell. Several mechanisms such as molecular mimicry, metabolic stress on beta cells, cryptic epitope exposure and costimulatory molecule upregulation have been proposed but not fully validated in the pathogenesis of T1D. Recently, T1D has been considered a consequence of dysregulated or inadequately developed regulatory immune responses in genetically predisposed individuals, similar to other autoimmune diseases.

The rapid increase in the incidence of T1D in developed countries during recent decades points to the role of environmental factors in this disease. Candidate environmental factors influencing T1D include various microbial and food components encountered at mucosal surfaces as well as gut mucosal parameters such as gut permeability.[89] The main difficulty in characterizing the environmental factors and mechanisms in T1D and possibly other autoimmune diseases is their complexity, the long lag period between the induction or disease-modifying events and the clinical onset of the disease, and the lack of studies in environmentally-defined, gnotobiological animal models. Furthermore, environmental factors in T1D seem to prevent full penetration of the disease rather than trigger it.

In non-obese diabetic (NOD) mice and biobreeding rats, the two well-established animal models of spontaneously developing autoimmune diabetes, the quality of specific pathogen-free housing facilities influences incidence of the disease. Animal facilities with positively defined specific pathogen-free microbiota (e.g., altered Schaedler microflora), antibiotic decontamination or rederivation of the breeding nucleus facilitate high diabetes incidence. Thus, clean conditions increase T1D incidence, whereas infections, including parasite infections and immunization with bacterial components, decrease the incidence.[90] We have shown rapid disease onset and 100% diabetes incidence in NOD females reared in germ-free conditions.[91] Wen et al. have also recently reported high diabetes incidence in germ-free mice and have documented an involvement of innate immune mechanisms in the disease. These findings indicate that some not yet well-defined components of the commensal microbiota exert diabetes-protective effects.[92]

The course of T1D may also be influenced by food. In both biobreeding rats and NOD mice, the diabetes-promoting agents are not carbohydrates but come mainly from the plant protein fraction of natural diets.[93] We have documented that a gluten-free diet, but also a diet with high gluten content, highly decreases diabetes incidence in NOD mice.[94,95] In addition, different mechanisms are responsible for

the disease prevention: some diets have a microbiota-dependent diabetes-protective effect, whereas others prevent T1D in a microbiota-independent manner.[91] Gliadin, the component of wheat gluten that triggers celiac diseases in susceptible individuals, was shown to activate innate immune mechanisms and to increase intestinal permeability.[83,96,97]

Increased gut permeability preceding clinical onset of the disease and signs of activation of the gut immune system were described in children with T1D and were related to the pathogenesis of this disease.[89] An impaired gut barrier function and subsequent consequences on the development of the disease were also demonstrated in the biobreeding rat model of T1D.[98] Thus, apart from various environmental factors acting in T1D, parameters of the gut mucosa forming an interface between the self and the environment further contribute to the complexity of the disease.

NEUROLOGICAL AND PSYCHIATRIC DISEASES
One of the most frequent and severe autoimmune neurological diseases is the demyelinating disease multiple sclerosis. The disease affects mainly young people, finally leading to their invalidity. Changes in gut barrier function, i.e., increased intestinal permeability in patients, as well as in their relatives, has been reported.[99] Viruses and bacteria have been suggested to participate in the pathogenesis of multiple sclerosis. Based on morphological and immunological findings in the brains of patients, attention has recently been given to the common infection with Epstein–Barr virus.[100] Bacterial involvement in the pathogenesis of multiple sclerosis was suggested after the bacterial peptidoglycan was found within antigen presenting cells (dendritic cells and macrophages) in the brains of patients but not of control individuals.[101] The potential role of molecular mimicry associated with infections was studied by Westall.[58] By comparing the sequences from three known encephalitogenic peptides with all known human bacterial and viral agents, this group found that mimics are present in a wide variety of microorganisms. Interestingly, the mimics were present predominantly in non-pathogenic gut bacteria.

Demyelination can be experimentally induced and achieved by immunization of mice with autoantigenic molecules isolated from central nervous system. This widely used model of multiple sclerosis, experimental autoimmune encephalomyelitis, has been invaluable in elucidating the pathogenesis of this debilitating disease and in creating new therapeutic approaches.[102] The role of the components of the microbiota in the pathogenesis of disease using this experimental model has recently been documented by Kasper's group, and the results of this study have been used to propose a novel treatment.[103,104]

The gut–brain axis is a bidirectional communication system through which the brain modulates gastrointestinal function and through which the gastrointestinal system is monitored by the brain. Neural and immunological and endocrine mechanisms are involved in this communication. The intestinal microbiota influences the gastrointestinal physiology, including the development and function of enteric nervous system.[20,105] The enteric nervous system (the 'second brain') directly controls the gastrointestinal tract functions. It consists of more neurons than there are in spinal cord (about 10^8), organized in myenteric and submucosal plexuses.[106] Interestingly, recent findings suggest a potential involvement of these structures in frequently occurring neurodegenerative disorders, like Parkinson's disease. Characteristic Lewy bodies, pathological hallmarks of Parkinson's disease, were found in intestinal biopsies of patients with Parkinson's disease.[107]

There is increasing evidence that the immune system, inflammation and mucosal barrier function are involved in the pathogenesis of some psychiatric diseases. Autism is an important mental illness and has attracted the attention of researchers due to its sharply increasing incidence in developed countries. Changes in antigenic load due to the impairment of gut barrier function were recently suggested as a triggering factor.[108] Autistic enterocolitis and changes in intestinal permeability were described in this early onset developmental disorder.[109] Moreover, urinary metabolic phenotyping has determined biochemical changes that were consistent with abnormalities in the composition of the gut microbiota found in autistic children.[110,111] Interestingly, in another mental illness, depression, 'leaky gut' has been suggested to play a pathogenic role: this assumption was based on findings of altered intestinal permeability in patients and their first-degree relatives.[112]

Analysis of behavioral changes in experimental animal models of neuropsychiatric diseases has started to be used to elucidate the role of the mucosal barrier function and the involvement of environmental factors in disease pathogenesis.[113] We have studied the behavioral changes occurring after induction of intestinal mucosal changes resembling celiac diseases by feeding of high doses of gluten to rats, and we found a higher emotionality in an open field test.[114] It is interesting to note that behavioral and psychological changes are often present in patients with active celiac disease, which is associated with findings of regional cerebral hypoperfusion in their brains.[115]

RHEUMATIC DISEASES
The involvement of intestinal changes in the pathogenic mechanisms of rheumatic diseases was suggested by findings of increased intestinal permeability and the presence of gastrointestinal symptoms in patients with juvenile idiopathic arthritis.[116] The frequent occurrence of arthritis in patients suffering from IBD suggests participation of the gut in this immune mediated rheumatic disorder.[117]

Infection with intestinal microbial pathogens such as *Salmonella*, *Shigella* and *Yersinia* precedes the development of reactive arthritis; these infections can trigger autoimmune reactions in joints.[118] Moreover, increased level of antibodies directed against antigens of certain species of gut bacteria (e.g. *Proteus*) suggests that there is an pathogenic relationship between these bacteria and rheumatoid arthritis.[119] Similarly, increased titers of anti-*Klebsiella* antibodies in patients with ankylosing spondylitis suggest that infection with this bacterium could be a triggering factor in these patients.[120] Only recently has the involvement of the gut microbiota community in the pathogenesis of rheumatic diseases been properly analyzed. Most studies involving the gut microbiota composition in rheumatoid arthritis have been performed using classical cultivation methods that do not allow analysis of the non-cultivable majority of gut microbiota. Studies based on the use of new molecular biological methods demonstrating alteration of the gut microbiota composition in patients with rheumatic diseases (e.g., juvenile arthritis) have appeared only recently.[121]

Animal models of rheumatic disease are frequently used, as in other diseases, to study pathogenic mechanisms and to develop new therapeutic approaches. Currently, these models are being used to study the participation of gut microbiota in disease development.[122] The rat HLA-B27 transgenic model of ankylosing spondylitis spontaneously develops this disease, associated with colitis, when reared in conventional conditions (i.e., with microbiota). After transfer into germ-free conditions, the transgenic rats lose inflammatory changes in the gut as well as in joints.[123] Alleviation of symptoms and inflammatory

changes through oral application of probiotics have been described in an experimental model of adjuvant-induced arthritis.[124] Exciting results were obtained from a recent study performed using a mouse model of rheumatoid arthritis, where it was demonstrated that a germ-free state decreases the clinical and autoimmune markers of arthritis. However, colonization with a unique non-cultivable bacterial strain belonging to mouse commensals, i.e., with segmented filamentous bacteria, induced the Th17 subpopulation, leading to clinical symptoms and increases in autoantibody production.[125]

OBESITY, CARDIOVASCULAR DISEASES AND ATHEROSCLEROSIS

In addition to the well-known role of intestinal bacteria in nutrition, commensal bacteria were found to play an important role in many physiological processes. Considerable interest in this role was generated by the findings of Jeffrey Gordon concerning changes in the expression of genes in germ-free mice following colonization by certain strains of intestinal bacteria.[7] These studies demonstrated significant effects of bacterial colonization on the expression of a wide range of genes, some of which are involved in metabolism.[50] The study from Backhed *et al.* examined the relationship between the composition of the microbiota and obesity.[126] Experimental models of genetically obese mice (leptin deficient ob/ob mice) and gnotobiological techniques (germ-free mice) were used in this study. These experiments demonstrated that the colonization of the intestine of germ-free mice by microbes from conventional mice led to a 40% increase in body fat over a relatively short period of time (2 weeks), despite the maintenance of low food intake. In other experiments, germ-free mice were colonized with the microbiota of obese mice and a control slim strain. Colonization with microbiota from obese mice induced a higher rise in body fat than did colonization with the microbiota from slim mice. The composition of the intestinal bacteria of the obese leptin deficient mice when analyzed by molecular biological methods was found to differ from that of the slim mice, particularly concerning the proportion of the two bacterial groups *Firmicutes* and *Bacteroidetes*: obese mice exhibited a 50% lower frequency of *Bacteroidetes* and an increased proportion of *Firmicutes*. These changes in the microbiota composition increased the ability to break down fiber into short chain fatty acids and to release additional energy that could be stored as fat.[126,127]

Interesting data have been generated in analyses of human microbiota. These results confirmed the data obtained in mice: obese patients had a lower proportion of *Bacteroidetes* and, if they lost weight during a year, the proportion of *Firmicutes* in their intestinal microbiota was comparable with that found in slim persons.[128] The recent study from Backhed *et al.* demonstrated that the colonization of germ-free mice leads to an increased *de novo* production of fat. This phenomenon was associated with lowered expression of the intestinal factor Fiaf, which takes part in the regulation of fat production.[126]

Many laboratories have endeavored to analyze the mechanisms by which intestinal bacteria affect the use of energy from food and to try to find bacterial strains whose administration would aid in the treatment of obesity, which puts the health of millions of people at risk for developing cardiovascular and other diseases.

Infections with *Chlamydia*, *Helicobacter pylori* or periodontopathic bacteria have been considered to increase the risk of development of cardiovascular disease.[52,129] Interest in the participation of the gut and its microbes was highlighted by findings demonstrating altered intestinal function, including increased permeability and augmented bacterial biofilm in patients with chronic heart failure.[130]

Experimental studies concentrated on the use of an advantageous mouse model of atherosclerosis, apolipoprotein E-deficient mice (ApoE$^{-/-}$). It has been demonstrated that infectious stimuli are not needed for the development of atherosclerotic plaques in ApoE-deficient mice fed by a high-cholesterol diet.[131] We also used this model, and have observed that, in contrast to the absence of atherosclerotic plaques in conventionally reared ApoE-deficient mice, germ-free ApoE-deficient mice consuming the same low-cholesterol standard diet exhibited developed atherosclerotic plaques in the aorta. Differences in the atherosclerotic plaques between germ-free and ApoE-deficient mice containing microbiota are not as apparent when the mice are fed by a high-cholesterol diet. These results document the protective effect of the microbiota on atherosclerosis development.[132]

ALLERGY

Epidemiological increases in the incidence of allergy, the most common chronic inflammatory disease, have occurred in recent years in economically developed countries and have triggered interest in potential environmental factors.[51,133] The search for an explanation for this trend resulted in the hypothesis that exaggerated hygienic conditions in these countries have decreased the quantity of natural infectious stimuli from the external environment, disturbing the well-balanced development of subpopulations of T cells, particularly the subpopulation of regulatory T cells ('hygiene hypothesis').[53,134,135] Recent microbiological analyses performed using classical and molecular biological techniques have demonstrated differences in the composition of intestinal microbiota between children from highly developed and underdeveloped countries. The former are born under controlled conditions in hospitals with maximal care and observance of hygienic measures. Consequently, the spectrum of microbes of their intestinal tract is much narrower than that of children from less developed countries.[34] Unfortunately, there is limited understanding of the role of intestinal lymphatic tissues and mucosal immunity in these processes. Recent studies have documented the changes in fecal microbiota in children suffering from food allergy.[136] Many efforts to influence the microbiotal composition of children in the early postnatal period have been attempted by the application of probiotics, and the results measured by allergy incidence later in life are promising.[137–139]

There are few experimental studies concerned with whether and how microbiota influences the development of allergy.[140] We have addressed the question of whether the intestinal microbiota affects the induction of mucosal (oral) tolerance against the birch pollen allergen. The Bet v1 allergen was applied intranasally or intragastrically in an experimental model of allergy induced by subcutaneous sensitization with the same allergen, and induction of tolerance was tested after application of an inducing allergen dose. Mice reared in germ-free and conventional environments did not differ in their ability to induce tolerance *via* the mucosal route or in their ability to induce a Th2 response. Therefore, we have demonstrated that the ability to induce mucosal tolerance is independent of the presence of microbiota in this model.[141]

CANCER

The involvement of infectious causes in the etiology of cancer has attracted the attention of researchers in recent years. At present, an association of cancer with bacterial and viral infectious agents is found in approximately 20% of all malignancies.[142] This is due to the increasing number of studies demonstrating the role of inflammation in establishing conditions that can deeply alter local immune responses

and, consequently, tissue homeostasis. In particular, inflammatory mediators such as IL-1, tumor-necrosis factor-α, IL-8, nitric oxide or prostaglandin-2 derivatives and molecules of the inflammatory pathways have been shown to be involved in a progressive interplay between immune cells and cells of a tissue undergoing transformation.[143] This association between inflammation and cancer has been highlighted in studies of IBD. The degree and prolongation of the duration of ulcerative colitis were recognized as factors leading to increased risk of gastrointestinal cancer development.[144,145] A correlation between gut microbiota composition and gastrointestinal cancers was examined in experimental animal models and clinical/epidemiological studies of environmental etiological factors. An association between a Western-style diet (red meat, fats and low vegetable intake) and changes in the composition of the gut microbiota has been observed in animal and human studies. This was linked to increased activities of fecal bacterial enzymes, as well as modification of sulfidogenesis and biliary acid metabolism with an impact on development of procarcinogenic conditions.[146,147] Interestingly, microbiota products can influence not only the local intestinal environment but also distant organs. Gut microbiota can metabolize certain plant-derived foods into biologically active compounds, e.g., enterolignans, that may play a role in carcinogenesis.[148] A recent meta-analysis indicated that these phytohormones may decrease the incidence of breast cancer.[149]

The highest production of carcinogens was associated with gut anaerobic bacteria and was lowered by supplementation with live lactobacilli.[150] *H. pylori* infection of the gastric mucosa was shown to create the conditions for developing ulcers, adenocarcinomas and gastric B-cell lymphomas. In fact, continuous inflammation induced by the bacteria activates cellular pathways inducing changes in mucin production (MUC2), as well as metaplasia and proliferation. Changes in mucin production and structure have been described both in gastric neoplastic conditions related to *H. pylori* and during the development of colorectal carcinoma.[151] These alterations progressively modify the relationship between the microbiota and the mucosal epithelia due to changes in the adhesiveness and integrity of the mucosal barrier. Some bacteria are able to induce modification of mucosal permeability, facilitating the translocation of bacteria and bacterial toxins (e.g., lipopolysaccharide). The inflammatory responses elicited were demonstrated to be able to enhance cancer progression.[152]

Bacteria represent a continuous stimulus for maintaining activated immunity in the gut mucosa and actively participate in the metabolism of bile and food components. Since germ-free mice lack this stimulus, they serve as a useful tool to study the role of bacteria in intestinal carcinogenesis. Compared with conventional mice, the incidence of both spontaneous and induced tumors is significantly lower in germ-free conditions.[153,154] Our studies of the participation of the microbiota in carcinogenetic processes were performed in the rat model of colorectal carcinoma and stressed the importance of the intestinal environment on the modulation of antitumor immunity. Compared to conventionally reared animals, germ-free rats develop fewer and smaller tumors. This result was associated with more active local and systemic immune responses.[155]

PROBIOTICS AND PREBIOTICS IN DISEASE PREVENTION AND THERAPY

Increased interest in the effects of the intestinal microbiota on human health has resulted in attempts to optimize the composition of the microbiota by dietary interventions or with probiotics, prebiotics or both (symbiotics).[156] The effects of probiotics depend on the properties of the microorganism used with both species- and strain-specific effects. Probiotics are mainly lactic acid bacteria (*Lactobacilli* and *Bifidobacteria*), but other bacterial species (enterococci or some strains of *E. coli*) and yeast have also been used as probiotics. Probiotic bacteria are often consumed in foods such as yogurts and cheese, in food supplements, or as drugs. Prebiotics are compounds that support the proliferation of beneficial bacteria (*Lactobacilli* and *Bifidobacteria*) in the intestine and include some saccharides (e.g., inulin). Probiotics have been shown to favorably influence the development and stability of the microbiota, inhibit the colonization by pathogens, influence the mucosal barrier by trophic effects on the intestinal epithelium, protect against physiological stress, and stimulate both specific and nonspecific components of the immune system.[139,157–163] Probiotics may well replace antibiotics whose resistance has been steadily increasing. Similar to the effects of microbiota, the effects of administration of probiotics and prebiotics are being intensively studied. Experiments using cell cultures and animal models are being performed to demonstrate the anti-inflammatory and immunomodulatory effects of different strains of probiotic microorganisms. To date, well-controlled clinical studies to clearly document the therapeutic or preventive effects of probiotics in various diseases are scarce. Even so, the therapeutic or preventive effects of certain probiotics have been documented in therapy of pouchitis, traveler's and antibiotic-associated diarrhea, irritable bowel syndrome and rotavirus enteritis.[164,165] The effects of probiotics in allergy prevention and therapy have been intensively studied, but the results are not yet conclusive.[166] A long-lasting effect on allergy prevention has been demonstrated in some studies.[137] The effects of probiotics on autoimmune and neoplastic diseases have been studied far less than the effects on allergy and intestinal diseases.[167,168]

Molecular mechanisms of probiotic effects in the intestine have begun to be elucidated in humans by analyzing local changes in their transcriptome.[169] The use of recombinant probiotic bacteria expressing a number of interesting biologically active molecules, such as allergens that could induce tolerance and inhibit allergic responses after administration in the gut, represents an exciting new direction.[170,171]

CONCLUSION

Just as homeostasis of our body systems is the product of many complex, redundant mechanisms, multigenic disease development is also dependent on both missing and overactivated pathways. The goal to find a common factor in the disease pathogenesis is difficult, genetic and pathophysiological data are incomplete, and the individual variability is enormous. Examination of the role of the microbiota in human illnesses using animal models of human diseases reared in defined (gnotobiotic) conditions could allow insight into the unusual complexity of the mechanisms involved in the initiation and maintenance of chronic diseases. Although the most important findings in this fascinating field are still to come, it is clear that our bacterial companions affect our fates more than previously assumed.

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4. GENERAL DISCUSSION

Mucosal immune system maintains a critical interface between the self and the outer environment. It has to balance induction of strong immune responses to danger signals as well as hyporesponsiveness or tolerance to non-danger stimuli such as dietary antigens or commensal microflora. In addition, it has to control these two phenomena not only locally but distribute the control (with some tissue related specificities) through the whole mucosal compartment. It is thus not surprising that mucosal immune system is important not only in local affections at mucosa such as in nose in case of nasal polyposis, but also in organ specific autoimmune disease, in which mucosal immune cells play an important role in pathogenesis of the disease but mechanisms of mucosal immune mechanism are also researched for the disease prevention or early cure.

In the first, more clinically oriented part of this theses we have investigated increased expression of chemokine receptors CCR1 and CCR3 in NP versus nasal mucosa. With respect to the hypothesis on the role of innate immune mechanisms and homeostasis of epithelial cells in NP we documented increased number of iNOS-positive and insulin-like growth factor-1 receptor (IGF-1R)-positive cells in both stroma and epithelium of NP.

Both pathogenesis and etiology of NP is not fully understood, what results in a lack of causative treatments. Experiments to uncover pathogenetic mechanisms and etiological agents are not easy to design, as there is no good experimental model of NP. It is therefore difficult to distinguish what it the causative mechanism and what is just an outcome of the pathological processes. Thus, no single agent or list of etiological agents has been identified in nasal polyposis. NP is considered at present as a multifactorial disease, in which the interplay of various environmental factors and genetic predisposition leads to its development.

Innate immune mechanisms, epithelial cells and above all the granulocyte infiltrate are most likely the major key players in the development of the disease. It is a bit surprising, that although there is a massive, eosinophilic and neutrophilic infiltrate present in the most common type of NP, these cell types have been a bit avoided or not adequately studied. For example, there is indeed a study assessing the number of Foxp3^{+} Tregs in nasal polyposis (Li et al., 2008), however, the functional role of eosinophils in the disease mechanisms has not yet been fully answered (Bachert et al., 2002; Fokkens et al., 2012; Bachert et al., 2015; Kato, 2015). This corresponds to basic immunology research, where methods evolved along the B and T cells, and NK cells, and only much later new functions

and mechanisms of action were discovered in eosinophils and neutrophils (Goldmann and Medinna, 2013). As regards the role of environmental factors, interaction of nasal mucosa with nasal microbiom deserves much more attentions (Hamilos, 2014; Chalermwatanachai et al., 2015). Last but not least, the definition of NP is rather old, based mainly on histology. With the rapidly advancing techniques of molecular immunology, there is a high need for a better definition of new (sub-) phenotypes of nasal polyposis (Bachert et al., 2015; Kato, 2015; Hamilos et al., 1995).

The second part of this thesis deals with the role of mucosal immunity and environmental factors (gluten-free diet and gliadin) in pathogenesis and prevention of type 1 diabetes.

Environmental factors (diets, microflora) play an important role in the recent increase of type 1 diabetes (T1D) in developed countries. Studies in both NOD mice and BB rats have documented that T1D is a diet-influenced disease (Scot, 1996; Coleman et al., 1990) and that a non-purified, gluten-free diet highly prevents development of diabetes in NOD mice (Funda et al., 1999; Schmid et al., 2004). Interestingly, there is published a case report of a newly diagnosed type 1 diabetic on gluten-free diet without need of insulin therapy (Sildorf et al., 2012). Because gluten-free (GF) diets highly prevent T1D in animal models we investigated the effect of dietary gluten on the mouse mucosal immune system. We also investigated innate signaling pathways of wheat proteins and tested intranasal gliadin as a vaccination strategy for prevention or even early cure of type 1 diabetes in NOD mice.

The mechanisms of environmental factors e.g. gluten-free diet are difficult to study because of the complexity of the topic. For example, the GF diet modifies the composition of gut microflora (Hansen et al., 2006), diabetes incidence in NOD mice is also highly influenced by intestinal microbiota as germ-free NOD females display 100 % diabetes incidence and rapid onset of diabetes (Wen et al., 2008; Funda et al., 2007). In addition, while diabetes preventive effect of some diets is microflora-dependent, the effect of the GF diet is not (Funda et al., 2007). In the light of these observations it is of interest that since diagnosis of individuals at high risk (2 or more autoantibodies) and rapid progression to clinical onset of diabetes is available at present, recruitment of such individuals for a prospective effect of gluten-free diet has started under the framework of the TEDDY (The Environmental Determinants of Diabetes in the Young; https://teddy.epi.usf.edu/index.htm) consortium.

Although type 1 diabetes is often reported as autoimmune disease it is good to keep in mind that it does not fulfill one of the important criteria of autoimmunity as defined by the

Witebsky and Rose (Rone and Bona, 1993); the disease-induction with a beta cell specific autoantigen has never been achieved in T1D. Immunizations with neither beta cell autoantigens nor pancreatic extract together with adjuvants were able to induce T1D (Chatenoud and Bach, 2005).

Several of the beta-cell autoantigens that have prevented T1D in animal models proceeded to human trials e.g. oral or intranasal insulin, s.c. and i.v. insulin or s.c. GAD65/alum (Diabetes Prevention Trial-Type 1 Diabetes Study Group, 2002; Baker et al., 2007; Wherrett et al., 2011). However none of these human trials has so far showed a protective effect. This is also case in other autoimmune diseases (Hanninen and Harrison, 2004). In addition, there is always a danger of induction of autoimmunity when using beta cell autoantigens for tolerance induction. Based on this evidence and also on the fact that environmental factors play important roles in the recent increase of T1D, we think environmental antigens related to T1D, such as intranasal gliadin and their mechanisms of action should be researched as novel prevention strategies.

For many years researchers were looking what is causing type 1 diabetes, yet there are several indications that in case of diabetes several environmental factors are rather preventing clinical onset of the disease in genetically predisposed individuals than actively causing it. Thus, a different view on the role of environmental factors has slowly evolved - it is perhaps not so relevant what factors are causing the type 1 diabetes, but rather what environmental factors prevented the full penetrance of the disease in the past and are not any more.   .

5. CONCLUSIONS

In the clinically oriented part of this thesis, we have focused on the role of mucosal immunity of the upper respiratory tract in disease conditions by studying possible pathogenic mechanisms of nasal polyposis - a very frequent diagnosis in ENT clinical practice, which pathogenesis as well as etiology is not resolved.

(A) We documented increased expression of chemokine receptors CCR1 and CCR3 in nasal polyps versus nasal mucosa. Increased number of CCR1 and CCR3 positive cells within nasal stroma and increased epithelial expression of CCR3 was showed in NP. These data document molecular basis for the increased migration and also accumulation of neutrophils and eosinophils in NP. Both CCR1 and CCR3 molecules may represent promising therapeutical targets in a local chronic inflammatory process such as NP.

(B) We studied the expression of insulin-like growth factor-1 receptor (IGF-1R) and inducible nitric-oxide synthase (iNOS) in nasal polyps and showed statistically significantly increased numbers of IGF-1R-positive and iNOS-positive cells in both stroma and epithelium of NP compared to nasal mucosa. These findings may have implications for the role of epithelial cell homeastasis and innate immune mechanism in pathogenesis of NP.

The second part of this thesis comprises experimental studies on the role of mucosal immunity and environmental factors in autoimmune diseases. More specifically, we studied the effect of environmental factors (diets, gliadin) on mucosal immune system of BALB/c mice and prevention of type 1 diabetes in the spontaneous NOD mouse model. We also addressed some innate mechanisms of gliadin actions.

(C)   Using immunocompetent BALB/c mice, we compared effects of the diabetes-preventive gluten-free diet and the standard, gluten containing diet on multiple regulatory T cell subsets and Th17 cells. We found diet-related changes in $\gamma\delta$ T cells, $CD4^+CD62L^+$ and $CD4^+CD45RB^{low}$ T cells, Th17 cells, but not in $CD4^+Foxp3^+$ Tregs, preferentially in mucosal lymphoid organs (MLN, PP, PLN). Our data thus show that the two tested diets -

diabetes permissive and diabetes preventive (in NOD mice) influence multiple regulatory T cell subsets and Th17 cells, especially in the mucosal lymphoid compartment.

(D) We assessed the cytokine profiles of polyclonally stimulated T cells in BALB/c mice fed the diabetes-preventive gluten-free diet and the standard, gluten containing diet. We found an altered, more proinflammatory cytokine signature associated with the diabetes-permissive, gluten containing standard diet in both mucosal but also systemic lymphoid organs. We think the shift towards a more proinflammatory cytokine profile may be directly related to the diabetes-permissive character of the standard diet.

(E) We investigated the innate signaling pathways of pepsin digest of gliadin and found that it leads to robust induction of IL-1β and IL-1α as well as some induction of IL-18 in PBMCs and monocytes from celiac disease patients. We then documented the IL-1β is induced via the MAPK-NF-κB pathway, the process is caspase-1 dependent and requires NLRP3 and ASC. Using various strains of KO mice we documented the TLR2/4/MyD88/TRIF signaling pathways are involved in the innate signaling by the pepsin digest of gliadin. Collectively, these data describe that innate immune pathways, such as TLR2/4/MyD88/TRIF/MAPK/NF-κB and NLRP3 inflammasome activation are involved in wheat proteins signaling.

(F) We tested a novel vaccination strategy in T1D by intranasal administration of an external environmental substance to NOD mice. I.n. application of gliadin led to significant prevention of diabetes and development of insulitis in NOD mice. It was even able to reduce diabetes incidence in prediabetic mice with advanced insulitis. We described changes in mucosal T cells, especially γδ T cells and their cytokine profiles. In conclusion, intranasal application of gliadin, an environmental antigen that has possible etilogocal influence in T1D, may represent a novel and safe approach to prevention or even early cure of T1D.

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